EXHIBIT A

Flt3 ligand (Flt3L), as a Vaccine Adjuvant, Results in the Generation of Antigen Specific IFNγ Producing T Cells after Immunization. Smorlesi, A., Shiota, F.M., and Disis, M. L. *Division of Oncology, University of Washington, Seattle, WA 98195.*

Flt3l is an important growth factor in the stimulation of dendritic cells (DC) from hematopoietic stem cells in vivo. Previous studies demonstrate cytokines which effect DC maturation and chemotaxis, such as GM-CSF, serve as excellent adjuvants in tumor antigen vaccine formulations. We evaluated the use of Flt3L as a potential vaccine adjuvant used in conjunction with a HER-2/neu (HER2) vaccine in a neu transgenic (neutg) mouse model. Neutg mice (FVB/n-MMTVneu-202) received daily treatment with Flt3L, 10 ug sq. for 10 days. A single immunization with HER2 protein was administered at different time points during the Flt3L cycle. One group of animals was immunized with 25ug HER2 protein on day 1 of the 10-day course of Flt3L (A), one group was immunized on day 5 (B), and a final group was immunized on the 10th day of the 10-day course of Flt3L (C). Mice vaccinated with HER2 protein and CFA were used as positive controls (D). Mice receiving FLT3 ligand alone with no HER2 immunization were negative controls. Immune spleen was analyzed for response to HER2 protein by measuring the amount of IL-4 or IFNγ release in response to challenge with antigen. Data is shown as the mean of 5 mice/group:

Groups	IL-4 pg/ml			IFNγ pg/ml		
Antigen	*None*	*HER2*	*PHA*	*None*	*HER2*	*PHA*
A	0	0	43	288	1939	3682
B	5	18	30	278	679	2909
C	20	21	31	254	345	2842
D	18	68	20	212	932	3407

HER2 IgG antibodies were detected in Groups B and D. Flt3L, when used as the sole adjuvant in a tumor antigen based vaccine, is a potent stimulator of an IFNγ producing antigen specific T cell response. The timing of antigen delivery in the course of Flt3L administration is important in generating immune responses.

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Cancer Vaccines: Novel Approaches and New Promise

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ABSTRACT. **Cancer vaccines are a promising tool in the hands of the clinical oncologist. We have summarized the most recent findings and achievements in this exciting field. Tumor-associated antigens, as a basis for the new cancer vaccines, are reviewed. We emphasize novel approaches for the design of safe and more effective vaccines for cancer. We also discuss the possible clinical applications and the future prospects for vaccine development.** PHARMACOL. THER. 81(2):121–139, 1999. **© 1999 Elsevier Science Inc. All rights reserved.**

KEY WORDS. Cancer vaccines, synthetic peptides, recombinant vaccines, antigen presentation, signal sequence, endoplasmic reticulum.

CONTENTS

ABBREVIATIONS. CDK, cyclin-dependent kinase; CEA, carcinoembryonal antigen; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ER, endoplasmic reticulum; GnT-V, *N*-acetylglucosaminyltransferase-V; HSP, heat shock protein; IFA, incomplete Freund's adjuvant; IL, interleukin; MART, melanoma antigen recognized by T-cells; MHC, major histocompatibility complex; MVA, modified vaccinia virus Ankara; RAGE, renal carcinoma antigen gene; rVV, recombinant vaccinia viruses; SRP, signal recognition particle; SSR, signal sequence receptor; TAA, tumor-associated antigens; TAP, transporter associated with antigen processing; VV, vaccinia virus.

1. INTRODUCTION

Immunotherapy may be classified into several types, including (1) active immunotherapy—specific stimulation of patient's immune system with vaccines, and/or nonspecific stimulation using adjuvants; (2) passive immunotherapy—treatment with exogenously produced antibodies; (3) adoptive immunotherapy—transfer of lymphocytes and/or cytokines; (4) restorative—designed to restore deficiencies in the patient's immune response; and (5) cytomodulatory—meant to enhance the expression of major histocompatibility complex (MHC) molecules on the surface of the tumor cells (Mitchell, 1993). Cancer vaccines exemplify active-specific immunotherapy, usually combined with adjuvants. Cancer vaccines initially were derived from mechanically disrupted, lysed, or irradiated tumor cells (Mitchell, 1995; Mitchell *et al.*, 1988). These whole cell-derived, polyvalent vaccines stimulate a wide spectrum of immune responses to a variety of tumor antigens, which is important in the immunotherapy of cancer. However, in order to clarify their mechanism of action and to attempt to improve these vaccines, it is necessary to refine them.

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Improved understanding of the molecular mechanisms of antigen processing and presentation, and the identification of tumor-associated antigens (TAA) in melanoma and other cancers, have allowed the development of specific vaccines. T lymphocytes recognize tumor antigenic epitopes—peptides bound to the MHC molecules. Each individual expresses as many as six different MHC Class I molecules (one A, B, and C allele from each parent). The variability is much greater for Class II molecules originating from three major subregions designated DP, DQ, and DR (Bodmer *et al.*, 1997). MHC Class I molecules are composed of a 45-kDa α-chain noncovalently associated with a 12-kDa β_2-microglobulin. On the other hand, MHC Class II molecules are composed of a 34-kDa α-chain that is noncovalently associated with a 28-kDa β-chain (Germain, 1994). Class II molecules present peptides of 12–25 amino acids, with a groove-contacting region in the middle and side chains of several amino acids (Rammensee *et al.*, 1995). Class II-binding peptides are generally of extracellular origin, and are predominantly recognized by CD4+ T lymphocytes (Rammensee, 1996). The cytotoxic T lymphocytes (CTLs) expressing CD8 molecules recognize Class I-restricted peptides of 8–10 residues, which are the products of intracellularly processed proteins (Rammensee, 1996; Yewdell and Bennink, 1992). Cytosolic peptides are

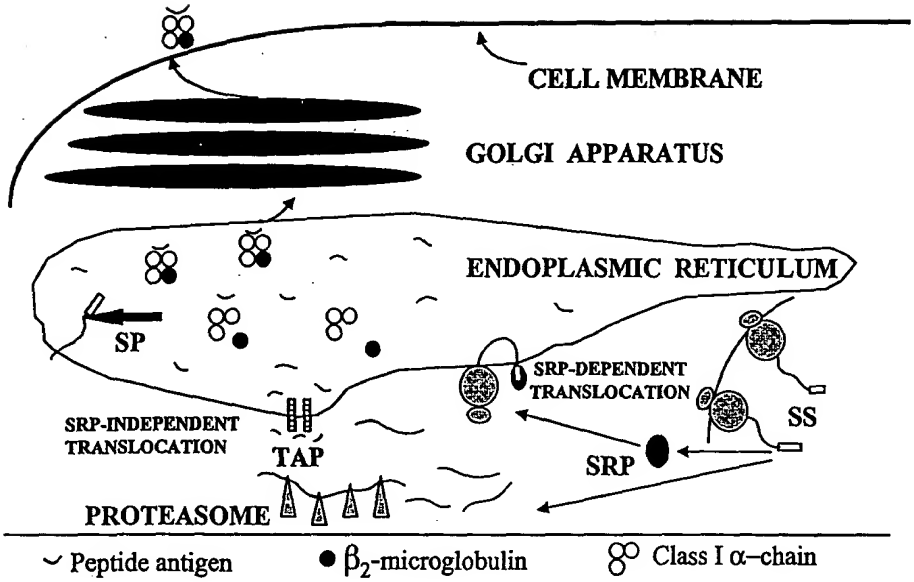

FIGURE 1. Mechanisms of processing and presentation of Class I-restricted antigens. SP, signal peptidase; SS, signal sequence.

transported across the endoplasmic reticulum (ER) membrane with the help of the ATP-dependent transporters associated with antigen processing (TAP) (Heemels and Ploegh, 1995; Spies *et al.*, 1992). Peptides complexed with Class I molecules in the ER are then transported to the cell surface for recognition by CTLs (Lehner and Cresswell, 1996; Yewdell and Bennink, 1992) (Fig. 1). The interaction between CTLs and the target tumor cells begins with the binding of the peptide antigen associated with the MHC Class I molecule to the T-cell antigen receptor. Lymphocyte-mediated cytolysis is further enhanced by accessory molecules, such as lymphocyte function antigen-1 and -3, co-stimulatory molecules (CD28, B7), and intercellular adhesion molecule-1 (Liu *et al.*, 1996).

2. TUMOR-ASSOCIATED ANTIGENS

The identification of several TAA in melanoma and other cancers has been an important development in the field of tumor immunology (Table 1). A variety of approaches have been used for the identification of TAA recognized by CTLs. Most of the melanoma antigens have been identified by screening cDNA expression libraries with CTLs reactive against melanoma. Two groups have used a transient expression system to identify cDNA pools that can sensitize target cells for recognition by T-cells. Individual cDNA clones have been isolated on the basis of their ability to stimulate cytokine release from specific CTLs after transfection into COS-7 cells or other appropriate targets. A number of melanoma antigens have been identified by this approach (Robbins *et al.*, 1994, 1995, 1996; R. F. Wang *et al.*, 1995; Boel *et al.*, 1995; Coulie *et al.*, 1994; Brichard *et al.*, 1993). Kawakami *et al.* used an HLA-A2+ breast cancer cell line transfected with a melanoma cDNA library to identify the genes encoding a melanoma antigen recognized by autologous T-cells (MART)-1 (Kawakami *et al.*, 1994a) and gp100 (Kawakami *et al.*, 1994b) antigens.

Another approach for the identification of TAA involves testing of known proteins for recognition by CTLs. With this approach Kawakami *et al.* (1993) found that the expression of tyrosinase and gp100 correlated with lysis by HLA-A2-restricted, melanoma-reactive CTLs. The same investigators demonstrated later that HLA-A2+ cell lines transfected with the gene encoding gp100 can be recognized by the melanoma-reactive CTLs (Bakker *et al.*, 1994; Kawakami *et al.*, 1994c). Tyrosinase gene product was also recognized by HLA-A2-restricted CTLs (Brichard *et al.*, 1993).

Synthetic peptides derived from the known target proteins could be used as vaccines. The specific binding of peptides to the MHC Class I molecules is characterized by a defined number of amino acids and two anchor residues. Crystallography studies revealed that the anchor residues represent interaction sites with the MHC binding grove. One anchor is at the C-terminus of peptides (usually position 9), while the second anchor is at position 2, 3, or 5 (Germain, 1995). Analysis of over 4000 peptides eluted from MHC molecules has identified over 100 motifs binding to a wide range of MHC molecules (Falk *et al.*, 1991; Brusic *et al.*, 1994). Although having the correct anchor residues is necessary for MHC binding, it is not sufficient, as non-anchor residues can exert important effects in both MHC peptide binding and T-cell stimulation. Therefore, it is very important to test both MHC binding and the ability of the peptide epitopes to stimulate appropriate CTL responses.

Direct isolation and sequencing of peptides eluted from the tumor cells is another method of identifying tumor-associated peptide antigens. Several groups have used this approach to isolate peptides recognized by melanoma-specific CTLs (Storkus *et al.*, 1993), as well as to sequence the peptides with a triple quadruple mass spectrometer (Cox *et al.*, 1994). This technique is complementary to the genetic approach because it allows measurement of the abundance of the antigenic peptides derived from the gene sequence.

TABLE 1. Human Tumor Antigens Recognized by T Lymphocytes

Antigen	MHC restriction	Peptide epitope[1]	Tumor type	Reference
MART-1/Melan-A	A2	AAGIGILTV	Melanoma	Kawakami *et al.*, 1994c; Coulie *et al.*, 1994
MART-1/Melan-A	A2	ILTVILGVL	Melanoma	Castelli *et al.*, 1995
gp100/Pmel-17	A2	YLEPGPVTA	Melanoma	Cox *et al.*, 1994
gp100/Pmel-17	A2	KTWGQYWQV	Melanoma	Kawakami *et al.*, 1995; Bakker *et al.*, 1995a
gp100/Pmel-17	A2	ITDQVPFSV	Melanoma	Kawakami *et al.*, 1995
gp100/Pmel-17	A2	VLYRYGSFSV	Melanoma	Kawakami *et al.*, 1995
gp100/Pmel-17	A2	LLDGTATLRL	Melanoma	Kawakami *et al.*, 1994b; Bakker *et al.*, 1995b
gp100/Pmel-17	A3	ALLAVGATK	Melanoma	Skipper *et al.*, 1996b
gp100	A24	VYFFLPDHL	Melanoma	Robbins *et al.*, 1997
Tyrosinase	A2	YMDGTMSQV	Melanoma	Skipper *et al.*, 1996a
Tyrosinase	A2	MLLAYLYCL	Melanoma	Wolfel *et al.*, 1994
Tyrosinase	A24	AFLPWHRLF	Melanoma	Kang *et al.*, 1995
Tyrosinase	B44	SEIWRDIDF	Melanoma	Brichard *et al.*, 1996
TRP1 gp75	A31	MSLQRQFLR	Melanoma	R. F. Wang *et al.*, 1995
TRP2	A31	LLPGGRPYR	Melanoma	Wang *et al.*, 1996
MAGE-1	A1	EADPTGHSY	Melanoma, other tumors	Traversari *et al.*, 1992
MAGE-1	Cw16	SAYGEPRKL	Melanoma, other tumors	van der Bruggen *et al.*, 1994b
MAGE-3	A1	EVDPIGHLY	Melanoma, other tumors	Gaugler *et al.*, 1994; Celis *et al.*, 1994
MAGE-3	A2	FLWGPRALV	Melanoma, other tumors	van der Bruggen *et al.*, 1994a
BAGE	Cw16	AARAVFLAL	Melanoma, other tumors	Boel *et al.*, 1995
GAGE-1,2	Cw6	YRPRPRRY	Melanoma, other tumors	Van den Eynde *et al.*, 1995
GnT-V	A2	VLPDVFIRC	Melanoma	Guilloux *et al.*, 1996
p15	A24	AYGLDFYIL	Melanoma	Robbins *et al.*, 1995
43-kDA Protein	A2	QDLTMKYQIF	Melanoma	Morioka *et al.*, 1994
MUM-1	B44	EEKLIVVLF	Melanoma	Coulie *et al.*, 1995
β-Catenin	A24	SYLDSGIHF	Melanoma	Robbins *et al.*, 1996
LB33-B	B44	EEKLIVVLF	Melanoma	Coulie *et al.*, 1995
CDK4	A2	ACDPHSGHFV	Melanoma	Wolfel *et al.*, 1995
HER-2/neu	A2	HLYQGCQVV	Ovarian, breast cancer	Disis *et al.*, 1994
HER-2/neu	A2	KIFGSLAFL	Ovarian cancer	Fisk *et al.*, 1995
HER-2/neu	A2	IISAVVGIL	Ovarian, breast, pancreatic and non-small cell lung cancer	Peiper *et al.*, 1997; Peoples *et al.*, 1995
HER-2/neu	A2	CLTSTVQLV	Ovarian cancer	Disis *et al.*, 1994
HER-2/neu	A2	VMAGVGSPYV	Ovarian cancer	Lustgarten *et al.*, 1997
CEA	A2	YLSGANLNL	Colon cancer	Tsang *et al.*, 1995

[1]Underlined letters represent mutated residues.

This is very important for recognition of the tumor cells by CTLs because at least 200 molecules of a peptide must occupy MHC Class I molecules in order for CTLs to lyse cancer cells (Christinck *et al.*, 1991). Another advantage of this technique is the direct identification of peptides naturally processed and presented on the tumor cell surface.

2.1. *Melanoma Antigens*

Human melanoma antigens can be classified into three groups: (1) antigens expressed in melanoma, normal melanocytes, and retina; (2) antigens expressed in several cancers and testis; and (3) antigens specific for individual tumors.

The first group consists of nonmutated shared tumor antigens. An interesting correlation between depigmentation of skin and hair and good clinical responses to chemo- and immunotherapy (Nordlund *et al.*, 1983; Richards *et al.*, 1992; Bystryn *et al.*, 1987) suggests that the same population of CTLs recognizes both melanoma antigens and nonmutated shared antigens on melanocytes. Rosenberg and White (1996) observed tumor regression in patients who developed vitiligo after interleukin (IL)-2-related immunotherapy, suggesting that autoreactive CTLs may be involved in tumor regression. Tyrosinase (Robbins *et al.*, 1994; Brichard *et al.*, 1993), MART-1/Melan-A (Coulie *et al.*, 1994; Kawakami *et al.*, 1994c), gp100 (Adema *et al.*, 1993; Kawakami *et al.*, 1994b), TRP1/gp75 (R. F. Wang *et al.*, 1995), and TRP2 (Wang *et al.*, 1996) have been identified as shared melanoma antigens recognized by CTLs (Table 1). More recently, Robbins *et al.* (1997) identified a nonmutated epitope encoded by an incompletely spliced gp100 gene transcript. These antigens may form the basis

for the development of effective vaccines, but their expression on normal tissues raises concerns about the possible development of immunological tolerance and autoimmunity associated with the immunotherapy.

The second group includes several families of antigens; specifically, MAGE, BAGE, GAGE, and RAGE. The MAGE genes are silent in a large panel of healthy adult tissues, with the exception of testis and placenta (Takahashi *et al.*, 1995; De Plaen *et al.*, 1994; van der Bruggen *et al.*, 1991). Brasseur *et al.* (1995) found that 48% of metastatic melanomas express MAGE-1 and more than 70% are positive for MAGE-2 or -3. Many other types of tumors have been found positive for the MAGE genes (Patard *et al.*, 1995; Weynants *et al.*, 1994). Like the MAGE genes, BAGE (Boel *et al.*, 1995) and GAGE (Van den Eynde *et al.*, 1995) genes are expressed predominantly in melanomas. A newly discovered gene called RAGE (renal carcinoma antigen gene) (Gaugler *et al.*, 1996) is also expressed in melanomas, sarcomas, and bladder tumors. Since these antigens are expressed in a variety of cancers, but not in healthy tissues, they may be appropriate targets for immunotherapy. However, peptide-specific CTLs were not detected in patients immunized with a MAGE-3-derived peptide, even though some tumor regression was observed (Marchand *et al.*, 1995).

Finally, some antigens unique to individual tumors appear through tumor-specific mutations, deletions, or recombination events. A point mutation might change a normal peptide not able to bind to MHC molecules into a peptide capable of binding to MHC and, therefore, presented to the immune system. Natural tolerance eliminates any CTLs recognizing normal peptides capable of binding to MHC. In the case of a point mutation, however, the modified peptide may become a target detected by existing CTLs. Several antigens generated by point mutations on a murine tumor were recognized by autologous CTLs (De Plaen *et al.*, 1988). Point mutations were also found to encode human tumor antigens recognized by CTLs (Robbins *et al.*, 1996; Coulie *et al.*, 1995; Wolfel *et al.*, 1995). For example, a single point mutation of the cyclin-dependent kinase (CDK)4 gave rise to a new T-cell epitope and generated a protein demonstrating decreased binding to the CDK4 inhibitor p16 (Wolfel *et al.*, 1995). An interesting finding was that a point mutation within the intronic region of a gene called MUM-1 was responsible for a new antigenic peptide recognized by HLA-B44-restricted CTLs (Coulie *et al.*, 1995). Since both the normal and the mutated peptides bound to HLA-B44, the altered amino acid appeared to represent a T-cell contact residue. Robbins *et al.* (1996) also described a mutation in the coding region of the β-catenin gene, resulting in a change in the amino acid sequence from a serine to a phenylalanine. This mutation creates an anchor residue, enabling the peptide to bind to HLA-A24. Since β-catenin is a cytoplasmic protein involved in cell adhesion, similar mutations may be associated with increased metastasis in melanoma and other cancers. In summary, this group of antigens should be recognized by melanoma-specific CTLs because their precursors should not have been depleted by the process of natural self-tolerance. From the clinical perspective, however, these antigens may not be useful for the development of cancer vaccines because of their restriction to very few individual tumors.

Using melanoma-specific CTLs, two nonmutated T-cell epitopes have been isolated recently. A transcript of the *N*-acetylglucosaminyltransferase-V (GnT-V) gene was isolated with an HLA-A2-restricted T-cell clone (Guilloux *et al.*, 1996). It was found that a T-cell epitope was encoded by a sequence located within one of the introns of the GnT-V gene. Although this product was expressed in about 50% of melanomas, it would be important to know if CTLs recognizing this epitope can be isolated from other melanoma patients. The other nonmutated epitope was identified by screening a melanoma cDNA library with HLA-A24-restricted tumor-infiltrating lymphocytes (Robbins *et al.*, 1995). It is not known yet whether this epitope could be used in a vaccine because the transcript of this gene product, called p15, was expressed in a wide variety of normal tissues.

Tumor-specific immune responses mediated by CD4+ T-cells have been demonstrated recently. Using Epstein-Barr virus-transformed B-cells, it was found that tyrosinase can also be recognized by CD4+ melanoma-reactive T-cells (Topalian *et al.*, 1994). Another group identified a separate tyrosinase-derived antigen recognized by both CD4+ and CD8+ effectors (Brichard *et al.*, 1993). Since CD4+ T-cells are essential for the effective antitumor immune response, these findings may help in the development of polyvalent cancer vaccines.

2.2. *Other Tumor-Associated Antigens*

In breast cancer and other adenocarcinomas, a polymorphic epithelial mucin has been characterized as a tumor antigen (Apostolopoulos and McKenzie, 1994; Ioannides *et al.*, 1993; Jerome *et al.*, 1991; Takahashi *et al.*, 1994; Magarian-Blander *et al.*, 1993; Gendler *et al.*, 1988). Mucins are high molecular weight glycoproteins. The MUC-1 mucin consists of a heavily glycosylated, tandemly repeating 20 amino acid sequence, specifically PDTRPAPGSTAPPAHGVTSA (Gendler *et al.*, 1988). Aberrant glycosylation of mucins on carcinomatous epithelial cells leads to the exposure of novel core epitopes that are recognized by cytotoxic T-cells (Magarian-Blander *et al.*, 1993). Even though HLA-unrestricted recognition of MUC-1 has been reported (Takahashi *et al.*, 1994; Magarian-Blander *et al.*, 1993), the establishment of mucin-specific cytotoxic T-cell lines (Ioannides *et al.*, 1993; Jerome *et al.*, 1991; Takahashi *et al.*, 1994) was a very important achievement in the attempt to develop cancer vaccines targeting this antigen. Since polymorphic epithelial mucin is much more highly expressed on carcinomas than on normal tissues, it could be a suitable target for immunotherapy (Apostolopoulos and McKenzie, 1994).

The HER2/neu proto-oncogene, expressed in breast cancer and other human cancers, encodes a tyrosine kinase

with homology to epidermal growth factor receptor, with a relative molecular mass of 185 kDa (Coussens *et al.*, 1985). HER2/neu protein is a receptor-like transmembrane protein comprising a large cysteine-rich extracellular domain that functions in ligand binding, a short transmembrane domain, and a small cytoplasmic domain (Coussens *et al.*, 1985). HER2/neu is amplified and expressed in many human cancers, largely adenocarcinomas of breast, ovary, colon, and lung. In breast cancer, HER2/neu overexpression is associated with aggressive disease and is an independent predictor of poor prognosis (Toikkanen *et al.*, 1992; Paik *et al.*, 1990). HER2/neu is considered a possible target for T-cell-mediated immunotherapy for several reasons:

- The protein is large (1255 amino acids) and, therefore, should contain epitopes appropriate for binding to most MHC molecules and thus, be potentially recognizable by all individuals;
- HER2/neu is greatly overexpressed on malignant cells and thus, T-cell therapy may be selective with minimal toxicity;
- The oncogenic protein is intimately associated with the malignant phenotype and with the aggressiveness of the malignancy, especially in breast and ovarian carcinomas (Slamon *et al.*, 1989; Toikkanen *et al.*, 1992; Paik *et al.*, 1990; Disis and Cheever, 1997).

Several Class I-restricted HER2/neu-derived peptides that were recognized by breast and ovarian cancer-specific CTLs have been described. The HLA-A2-restricted peptides p48–56 (HLYQGCQVV) (Disis *et al.*, 1994) and p369–377 (KIFGSLAFL) (Fisk *et al.*, 1995) were derived from the extracellular domain of the HER2/neu protein. Another HLA-A2-restricted peptide is located in the transmembrane portion of the same protein p654–662 (IISAV VGIL) (Peoples *et al.*, 1995). In addition, the peptide p789–797 (CLTSTVQLV) (Disis *et al.*, 1994) was derived from the intracellular domain of the HER2/neu protein. Using double transgenic mice expressing both HLA-A2.1 and human CD8 molecules, the peptide p773–782 (VMA GVGSPYV) was identified recently (Lustgarten *et al.*, 1997).

A number of research groups were tempted to speculate that T-cells would recognize peptide products of mutated oncogenes or tumor-suppressor genes. Indeed, T-cells recognizing peptides derived from mutated forms of ras (Jung and Schluesener, 1991; Peace *et al.*, 1993) and p53 (Houbiers *et al.*, 1993; Yanuck *et al.*, 1993) have been identified. In most cases, however, these T-cells did not recognize tumor cells expressing the relevant mutated protein, which raises the important question whether mutated proteins are normally processed and presented at the tumor cell surface.

3. NOVEL VACCINE APPROACHES

3.1. *Peptide Vaccines*

The identification of peptide sequences recognized by CTLs has led to attempts to directly induce CTL responses *in vivo* (Cormier *et al.*, 1997; Schulz *et al.*, 1991; Aichele *et al.*, 1990). Successful immunization of mice has been accomplished with peptides formulated with immunostimulating complex (Lipford *et al.*, 1993), entrapped in liposomes (Zhou *et al.*, 1992), osmotically loaded into syngeneic splenocytes (Zhou *et al.*, 1992), or coated on their surface (Harty and Bevan, 1992). Synthetic viral peptides covalently linked to a lipophilic compound were also capable of inducing a CTL response (Deres *et al.*, 1989). Effective immune responses were also elicited in mice with a mutant p53 peptide in adjuvant (Noguchi *et al.*, 1994) or with either mutant or wild-type p53 peptides loaded on dendritic cells (Mayordomo *et al.*, 1996). We showed in two murine antigenic systems that fusion peptides with an synthetic ER signal sequence at the NH_2-terminus of the minimal peptide were more effective than the minimal peptide alone in generating specific CTL responses (Minev *et al.*, 1994). Furthermore, we found that the CTL response was MHC Class II independent, could not be attributed to increased hydrophobicity of the fusion peptides, and was very effective in prolonging the survival of tumor-challenged mice.

A number of peptide antigens expressed on melanoma and other human cancers have been discovered (Gendler *et al.*, 1988; Fisk *et al.*, 1995; Disis *et al.*, 1994; Maeurer *et al.*, 1996b; Kawakami *et al.*, 1996) (Table 1). Peptide vaccination of cancer patients has been limited to very few trials so far. Immunizations with MAGE-3-derived peptide without any adjuvant induced limited tumor regressions in 5 of 17 patients with melanoma (Marchand *et al.*, 1995). Salgaller *et al.* (1995) reported generation of CTLs specific for one of three gp100-derived peptides in patients vaccinated with peptide in incomplete Freund's adjuvant (IFA). Immunization of three patients with advanced melanoma with peptide-pulsed autologous antigen-presenting cells led to induction of peptide-specific CTLs (Hu *et al.*, 1996; Mukherji *et al.*, 1995). The peptide used in this study was derived from MAGE-1 and was restricted to HLA-A1.1. The lack of any therapeutic response observed in this trial might be explained by the advanced stage of the disease in these patients. In another study, 9 melanoma patients were vaccinated weekly for 4 weeks with a combination of peptides derived from the MART-1, tyrosinase, and gp100 proteins (Jager *et al.*, 1996a,b). Successful immunization against peptides could be detected *in vitro* in two of six patients against the tyrosinase peptide, three of six patients against the MART-1 peptide, and none of six patients receiving the gp100 peptide. More recently, 18 patients with melanoma were immunized with a peptide derived from MART-1, emulsified with IFA (Cormier *et al.*, 1997). An enhancement of cytotoxic activity against MART-1 was detected with minimal toxicity for the patients consisting of local irritation at the site of vaccination. Serial administrations of this peptide appeared to boost the level of cytotoxicity *in vitro*, although clinical regression of the tumor was not observed. In another trial, patients with advanced pancreatic carcinoma were vaccinated with a synthetic ras peptide pulsed on antigen-presenting cells isolated from peripheral

blood (Gjertsen *et al.*, 1996). This procedure led to generation of cancer cell-specific cellular response without side effects. However, in all patients, tumor progression was observed after the vaccination.

Several strategies for modifying peptides have been attempted in order to improve their efficacy as cancer vaccines. The clinical use of peptides is limited by their rapid proteolytic digestion. To overcome this limitation, Celis *et al.* (1994) designed a peptide construct containing a pan-reactive DR epitope, a CTL epitope, and a fatty acid moiety. A lipopeptide-based therapeutic vaccine was able to induce strong CTL responses both in humans and in animals (Vitiello *et al.*, 1995). Several studies demonstrated a correlation between MHC binding affinity and peptide immunogenicity (Lipford *et al.*, 1995; Chen *et al.*, 1994; Sette *et al.*, 1994). Peptides derived from gp100, whose anchor residues were modified to fit the optimal HLA-A2 binding motif, stimulated tumor-reactive CTLs more efficiently than the natural epitopes (Lipford *et al.*, 1995; Parkhurst *et al.*, 1996). An unmodified, gp100-derived peptide failed to elicit peptide-specific CTLs in melanoma patients after subcutaneous administration with IFA (Rosenberg *et al.*, 1998). In contrast, vaccination with the modified peptide induced CTL responses in 91% of cases. None of the 11 patients immunized with the modified peptide in IFA alone experienced an objective tumor response. Interestingly, administration of the modified peptide along with high-dose IL-2 led to a clinical response rate of 42% in a group of 31 patients. HER-2/neu-derived peptides were also evaluated for their ability to bind to MHC Class I molecules *in vitro* (Fisk *et al.*, 1995). It was found that amino acid substitutions at positions 1 and 9 could improve the HLA binding of these peptides without interfering with recognition by HER-2/neu-specific CTLs. An interesting strategy was used to enhance the immunogenicity of a peptide derived from human carcinoembryonal antigen (CEA) (Zaremba *et al.*, 1997). Four amino acid residues in this peptide, predicted to interact with the T-cell receptor, were replaced. This change led to more efficient sensitizing of CTLs yet was unassociated with improved binding to HLA-A2 molecules. These CTLs were shown to recognize both the original and the modified peptide, as well as human tumor cells expressing CEA. Therefore, amino acid modifications optimizing the binding of the peptides to MHC molecules, or their interactions with the T-cell receptor, might be useful in designing peptide vaccines for cancer.

We investigated the effectiveness of several synthetic insertion signal sequences in enhancing the presentation of the HLA-A2.1-restricted melanoma epitope MART-1_{27-35} (Minev *et al.*, 1996). An important step in presentation of the Class I-restricted antigens is the translocation of processed proteins from the cytosol across the ER membrane mediated by TAP proteins or as an alternative, by ER-insertion signal sequences located at the NH_2-terminus of the precursor molecules (Voigt *et al.*, 1996; Anderson *et al.*, 1991; Hunt *et al.*, 1992; Rapoport, 1992) (Fig. 1). Insertion signal sequences consist of three regions, with specific characteristics shared by both eukaryotes and prokaryotes (Gierasch, 1989): (1) a basic NH_2-terminal region consisting of 1–3 positively charged residues participating in binding and orienting the signal sequence with respect to the membrane surface, (2) a central hydrophobic region of 8–12 hydrophobic residues, and (3) a polar carboxy-terminal region of 5–7 residues with higher average polarity than the hydrophobic region. The amino acids in this region contribute to the predicted β-turn that is found immediately before or after the cleavage site. The β-turn is thought to be important for peptidase access to the cleavage site (von Heijne, 1986). Using a technique known as osmotic lysis of pinocytic vesicles, we loaded several synthetic peptide constructs into the cytosol of antigen processing-deficient T2-cells, TAP-expressing human melanoma cells, and dendritic cells (Moore *et al.*, 1988; Okada and Rechsteiner, 1982). We examined whether the natural signal sequences ES (derived from the adenovirus E3/19K glycoprotein) (Persson *et al.*, 1980) and IS (derived from interferon-β) (Houghton *et al.*, 1980) could enhance and prolong presentation of MART-1_{27-35}. We found that the addition of signal sequence at the NH_2-terminus, but not at the COOH-terminus, of MART-1_{27-35} greatly enhanced its presentation in both TAP-deficient and TAP-expressing cells. A newly designed peptide construct composed of the epitope replacing the hydrophobic part of a natural signal sequence was also effective. Interestingly, an artificial signal sequence containing the epitope was the most efficient construct for enhancing its presentation. These peptide constructs facilitated epitope presentation in a TAP-independent manner when loaded into the cytosol of TAP-deficient T2-cells. In addition, loading of these constructs into TAP-expressing melanoma cells also led to a more efficient presentation than the loading of the minimal peptide. Importantly, loading of human dendritic cells with the same constructs resulted in a prolonged presentation of this melanoma epitope. These findings may be of practical significance for the development of synthetic anticancer vaccines and *in vitro* immunization of T_{CD8+} for adoptive immunotherapy.

In this study, as in our previous findings in two murine antigenic systems (Minev *et al.*, 1994), we concluded that the position of the signal sequence relative to the MHC Class I-restricted epitope was critical to its ability to facilitate antigen presentation. NH_2-terminal localization of the signal sequence is advantageous for antigen presentation, probably because the signal peptidase requires this configuration in order to cleave off the minimal peptide in the ER. This possibility was suggested in a study showing that TAP-deficient T2-cells could be sensitized to lysis by CTLs when infected with recombinant vaccinia viruses (rVVs) expressing signal sequences situated at the NH_2-terminus, but not at the COOH-terminus of the minimal peptides (Bacik *et al.*, 1994). rVVs were also utilized by Restifo and co-workers for effective generation of specific CTL responses *in vivo* (Irvine *et al.*, 1995; Restifo *et al.*, 1995). In these studies, linking of a signal sequence to the NH_2-terminus of the

peptide epitope had a distinct advantage over the minimal peptide alone.

In this set of experiments, we utilized post-translational translocation of fusion peptides through the ER membrane. For the vast majority of proteins, translocation across mammary ER membrane has a strict requirement for co-translational delivery of the nascent chain (Walter and Johnson, 1994; Rapoport *et al.*, 1996). Post-translational translocation can occur not only across bacterial (Bassford *et al.*, 1991) and yeast membranes (Brodsky, 1996), but also across mammalian ER membranes (Perara *et al.*, 1986; Muller and Zimmermann, 1988; Schlenstedt *et al.*, 1990). These studies suggest that both co-translational signal recognition particle (SRP)-dependent and post-translational SRP-independent pathways coexist in eukaryotes. For post-translational translocation, the substrate protein must be retained in the cytosol in a translocation-competent state, i.e., be prevented from folding, aggregating, or precipitating. Thus, it seems plausible that only proteins that can use the SRP-independent translocation pathway efficiently have evolved signal sequences that allow them to bypass SRP.

The signal sequence at the amino-terminus may enhance the translocation of the minimal peptide due to the higher hydrophobicity or higher resistance of fusion peptides to proteolytic enzymes. However, in our experiments, loading of the T2-cells with constructs with carboxy-terminal location of the signal sequence, whose hydrophobicity was determined to be nearly identical to that of the regular constructs, was not efficient (Chavez *et al.*, 1996). It is also possible that the signal sequence in our constructs bound directly to the signal sequence receptor (SSR), which is an integral glycosylated protein of the ER membrane (Wiedmann *et al.*, 1987). The basic mechanism of targeting may be the interaction of a signal sequence with SSR, whereas the function of SRP may be to prevent folding of the polypeptide into an unfavorable conformation. With proteins that are transported post-translationally across the ER membrane, the conformation of the polypeptide may allow an interaction with the SSR. In support of this is the finding that the SSR resides in the immediate vicinity of the signal sequence of the nascent chain during the initial SRP-dependent targeting event (Wiedmann *et al.*, 1987).

We exploited the hydrophobic nature of MART-1_{27-35} and demonstrated that it could be presented on the surface of T2-cells when incorporated into a signal sequence. In agreement with our finding, Gueguen *et al.* (1994) showed with recombinant vaccinia vectors that a peptide derived from a signal sequence cleaved in the ER can provide an epitope for HLA-A2-restricted T-cell recognition in the TAP-deficient T2-cells. The ER is a proteolytically active environment, capable of generating Class I binding peptides (Elliott *et al.*, 1995; Hammond *et al.*, 1993). On the other hand, two studies found that functional TAP was required for efficient presentation of Class I-restricted epitopes from influenza (Uger and Barber, 1997) and lymphocytic choriomeningitis virus (Hombach *et al.*, 1995) incorporated into signal sequences. This suggests that in some cases, cytosolic degradation rather than ER proteolysis is responsible for generating the Class I binding epitopes. It seems likely that different fusion proteins fold differently in the cytosol, and some of them may assume a translocation-unfavorable state and are degraded in the cytosol.

We also found a very efficient presentation of MART-1_{27-35} loaded into TAP-expressing tumor cells and dendritic cells (DCs), which may be explained by the availability of intact TAP transporters in these cells. In this case, some of the loaded MART-1_{27-35} may have been translocated by TAP from the cytosol even 8 days after loading. The size of MART-1_{27-35} (9 amino acids) is appropriate for optimal translocation by TAP (Heemels and Ploegh, 1995). Still, fusion peptides were more effective than MART-1_{27-35}, probably because of their translocation by both TAP-dependent and TAP-independent pathways. The later mechanism of peptide translocation may be important for antigen presentation, especially in cancers that fail to utilize the classical MHC Class I pathway (Khanna *et al.*, 1994; Seliger *et al.*, 1996; Cromme *et al.*, 1994; Restifo *et al.*, 1993; Maeurer *et al.*, 1996a; Ferrone and Marincola, 1995). From a clinical perspective, immunization with peptides may be preferable to immunization with rVVs because of its safety and because it is not associated with diminished immune responses in patients immunized against smallpox. Immunizing with minimal determinant constructs may avoid the possible oncogenic effect of full-length proteins containing ras, p53, or other potential oncogenes. In addition to their safety, peptide vaccines can be designed to induce well-defined immune responses and can be synthesized with very high purity and reproducibility in large quantities. Another potential advantage of peptide vaccines over whole proteins or DNA vaccines is the ability to identify the specific epitopes of the tumor antigens to which an individual is able to mount an immune response, but not a state of immune tolerance (Celis *et al.*, 1995). In addition, *in vivo* or *in vitro* immunization with peptide antigens "packaged" in DCs or other antigen-presenting cells (discussed in Section 3.4) opens an exciting opportunity for eliciting powerful CTL responses.

A disadvantage of peptide vaccines is their poor immunogenicity and monospecificity of the induced immune response. Another limiting factor for the use of peptide vaccines in outbred populations is that T-cells from individuals expressing different MHC molecules recognize different peptides from tumor or viral antigens in the context of self-MHC. However, the use of synthetic peptides from TAAs that are presented by common MHC molecules may overcome this problem. Poor immunogenicity caused by rapid degradation of the peptides by serum peptidases may be corrected by modifications or incorporation of the peptides into controlled release formulations.

3.2. *Recombinant Viruses*

Many different viruses have been used to construct recombinant vaccines. These vaccines have the advantage of in-

ducing both humoral and cell-mediated immune responses, in some cases after a single application. However, possible disadvantages of recombinant viruses include recombination with wild-type viruses, conversion to virulence, oncogenic potential, or immunosuppression. We will briefly discuss current strategies to overcome some of these obstacles in order to develop efficient recombinant viral vaccines.

VV was demonstrated to be a safe and very effective immunogen in the smallpox eradication campaign, where it was administered to over 1 billion people. Large amounts of foreign DNA can be stably inserted into the VV genome by homologous recombination (Moss, 1991). Another advantage of this vector is a very efficient post-translational processing of the inserted genes within host cell cytoplasm. The induction of potent cellular and humoral immune responses with rVVs was observed in several tumor model systems (Hodge *et al.*, 1995; Bronte *et al.*, 1995; Irvine *et al.*, 1995; Restifo *et al.*, 1994). VV encoding human CEA induced both humoral and cellular immune responses when given to colorectal cancer patients (Tsang *et al.*, 1995). Significant advances in the understanding of the molecular mechanisms of antigen processing have led to the construction of a new generation of recombinant viruses. Viruses containing minigenes encoding short antigenic peptides bypassed the need for proteolysis of the antigenic proteins (McCabe *et al.*, 1995). ER-insertion signal sequence added to the amino terminus of the minigenes circumvented the requirements for both proteolysis and transport of the antigenic peptides (Bacik *et al.*, 1994; Irvine *et al.*, 1995; Restifo *et al.*, 1995). It was also possible to engineer VVs able to enhance MHC Class II presentation of a tumor antigen for optimal recognition by CD4+ T lymphocytes (Lin *et al.*, 1996; Wu *et al.*, 1995).

However, due to the induction of high titers of antivaccinia antibodies, rVVs may be given only once or twice (Kundig *et al.*, 1993; Etlinger and Altenburger, 1991). It was demonstrated that intratumoral inoculation of VV induced very high levels of antivaccinia antibodies in serum. Surprisingly, however, it was possible to sustain viral gene function by repeatedly injecting vaccinia into the tumor site (Mastrangelo *et al.*, 1995). Other studies also showed effective generation of B- and T-cell responses to the products of rVVs (Graham *et al.*, 1993; Cooney *et al.*, 1991). A promising new strategy to increase the efficacy of recombinant viral vaccines is to use two different vectors for priming and boosting vaccinations (Irvine *et al.*, 1997). This approach was much more effective in generating antigen-specific CTL responses than the use of one vector for both priming and boosting.

Since vaccinia is a replication-competent virus, it may cause disseminated viremia, especially in immunosuppressed individuals (McElrath *et al.*, 1994). Therefore, several research groups attempted to develop recombinant vaccines based on nonreplicating viruses (Somogyi *et al.*, 1993; Baxby and Paoletti, 1992). Utilizing recombinant fowlpox virus, which does not replicate in mammalian cells, M. Wang *et al.* (1995) were able to treat established tumors in mice. An important aspect of this work was the finding that prior immunization with VV did not abrogate the immune responses elicited by the recombinant fowlpox virus. Another group reported protective immune responses generated by recombinant fowlpox viruses against rabies glycoprotein and experimental tumor (Taylor *et al.*, 1988). A different nonreplicating virus, canarypox virus (ALVAC), was used to generate recombinant viruses able to elicit immune responses against a variety of antigens (Cox *et al.*, 1993; Cadoz *et al.*, 1992). Roth *et al.* (1996) reported protection of mice from tumor cells expressing p53 using an ALVAC-p53 construct. More recently, Hodge *et al.* (1997) evaluated the efficacy of a recombinant canarypox virus expressing human CEA, alone or in combination with recombinant vaccinia-CEA. They found that an immunization scheme using a recombinant VV followed by recombinant canarypox virus was far superior than the use of either one alone in eliciting CEA-specific immune responses. Importantly, multiple boosts of recombinant canarypox virus following immunization with recombinant vaccinia further potentiated the antitumor effects and T-cell responses. A clinical trial with vaccinia-CEA in patients with colorectal cancer resulted in eliciting of cell-mediated immune responses against CEA-derived peptide (Tsang *et al.*, 1995). In this study, rejection of the VV itself was not observed. A nonreplicating VV, known as modified VV Ankara (MVA), is avirulent in normal and immunosuppressed animals, and was shown to have no significant side effects after inoculation of 120,000 humans (Meyer *et al.*, 1991). Since replication of MVA is blocked at a step of virion assembly (Sutter and Moss, 1992), rather than at an early stage, MVA vectors produce recombinant proteins in amounts similar to those of wild-type viruses. In addition, the immunogenicity of MVA recombinants in mice is similar to that of virulent strains (Sutter *et al.*, 1994). Therefore, MVA is a very promising vector for development of recombinant vaccines for cancer.

3.3. *DNA Vaccines*

This novel approach involves direct inoculation of expression plasmids, which results in the induction of long-lasting immune responses against the expressed antigens. Fynan *et al.* (1993) compared six routes of inoculation of naked DNA for their relative efficacies. In this study, intramuscular injection of DNA generated the best response, whereas inoculation of DNA-coated gold particles using a "gene gun" required significantly lower doses of DNA. It was found that the uptake of the injected DNA is an active energy-dependent process (Wolff *et al.*, 1992a). Once inside the cell, plasmid DNA can get through the nuclear membrane and persists as a nonreplicating episomal molecule, which explains the long-lived foreign gene expression (Wolff *et al.*, 1992b). The low, but long-lasting, expression of the encoded antigens is an important feature of this approach (Yankauckas *et al.*, 1993). The duration of expression seems to be more important than the dose of the an-

tigen for induction of CTL responses, although DNA immunization has been shown to result in both cellular and humoral immune responses, and in generation of antigen-specific CD8+ and CD4+ T-cells (Fuller and Haynes, 1994; Pertmer *et al.*, 1995). Irvine *et al.* (1996) reported effective treatment of established pulmonary metastases, using a "gene gun" for DNA immunization. In this study, recombinant cytokines enhanced the therapeutic effects of this approach. Enhancement of the immune response against a model tumor antigen was also observed after co-transfection of the genes coding for the cytokine granulocyte/macrophage colony-stimulating factor and the co-stimulatory molecule B7-1 (Conry *et al.*, 1996). Several elegantly designed studies addressed the important question of the mechanism of DNA immunization (Corr *et al.*, 1996; Ulmer *et al.*, 1996; Condon *et al.*, 1996). Results demonstrate that the antigen-presenting cells (APCs) can be transfected directly or they can acquire the antigens expressed by other transfected cells. However, only professional APCs are able to initiate primary immune responses as a result of DNA immunization. These findings are extremely important in the development of DNA-based vaccines for clinical application. A promising DNA vaccine has been developed against a B-cell lymphoma (Syrengelas *et al.*, 1996). Another plasmid for clinical application encodes human CEA and the hepatitis B virus surface antigen, each of them under a separate cytomegalovirus promoter (Conry *et al.*, 1996).

DNA vaccines have several potential advantages over peptide and recombinant viral vaccines. DNA vaccines are simpler and cheaper to produce. DNA immunization is not associated with an anamnestic immune response, which is responsible for the rapid clearance of viral constructs. Another major advantage is that DNA vaccination induces very long-lasting immune responses. Addressing a major concern for the clinical use of DNA-based vaccines their safety, Kurth (1995) calculated that the probability of tumor-promoting events by plasmid DNA integration was below that of the statistical events leading to a mutation within the lifetime of an individual. In all, DNA-based vaccines seem to be a promising approach for the treatment of cancer.

3.4. *Dendritic Cells*

Recently, several groups reported the successful use of DCs for inducing antitumor immune responses in both animals and humans. DCs are the most potent APCs for the initiation of antigen-specific immune responses (Cumberbatch *et al.*, 1991; Steinman, 1991). In addition to their ability to efficiently acquire and process antigens (Nijman *et al.*, 1995; Sallusto *et al.*, 1995), DCs express high levels of MHC Class I and Class II molecules, as well as co-stimulatory molecules (Kiertscher and Roth, 1996; Zhou *et al.*, 1995) essential in antigen presentation. Therefore, many investigators attempted to immunize with peptide-pulsed DCs. It was found that immunization with peptide-pulsed DCs is superior to injection of peptide in adjuvant in inducing potent cytotoxic T-cell responses (Porgador and Gilboa, 1995). A similar strategy was also reported by others to be successful in eliciting T lymphocyte-mediated protective antitumor immunity (Ossevoort *et al.*, 1995; Mayordomo *et al.*, 1995; Celluzzi *et al.*, 1996). A set of experiments in a mouse model showed that DCs injected i.v. traveled to the major lymphoid organs. IL-12 and the co-stimulatory pathway, dependent on the receptors B7/CD28, were important for the induction of an effective immune response (Mayordomo *et al.*, 1995). A possible disadvantage of peptide pulsing is the short half-life (2–10 hr) of most MHC-restricted epitopes (Eberl *et al.*, 1996), which creates the requirement for several injections of peptide-pulsed DCs to achieve effective immune responses (Zitvogel *et al.*, 1996; Mayordomo *et al.*, 1995; Celluzzi *et al.*, 1996). Therefore, development of different methods for loading of antigens allowing DCs to utilize their own intracellular pathways is highly desirable. The antigens of interest must be present in the cytosol of the DCs in order to enter the intracellular pathway, leading to their loading onto MHC Class I molecules and the subsequent activation of CD8+ T-cells. Surprisingly, Paglia *et al.* (1996) were able to prime murine CTLs against tumor antigen by incubating DCs with whole protein *in vitro*. Another group reported the generation of specific CTLs in mice vaccinated with DCs pulsed with RNA from an ovalbumin-expressing tumor (Boczkowski *et al.*, 1996). In this approach, however, rapid degradation of RNA limits the duration of antigen expression. A recent study showed that treatment of pulmonary metastases in mice with bone marrow-derived DCs transduced with retroviral vector encoding a model antigen was very effective (Specht *et al.*, 1997). The reduction of the metastatic nodules was associated with induction of antigen-specific CTLs. Adenovirus vectors were also used to transduce DCs with genes coding for tumor antigens. It was demonstrated in a murine breast cancer model that a single injection with transduced DCs provided complete protection against tumor cell challenge (Wan *et al.*, 1997). This approach was not limited by hepatic toxicity and the development of neutralizing antibodies associated with the direct administration of the adenoviral vectors (Fisher *et al.*, 1997; Yang *et al.*, 1995). A recent study also suggested that adenovirus vectors are a promising vehicle for genetical engineering of human DCs (Arthur *et al.*, 1997). A comparison of various gene transfer methods in human DCs showed that adenovirus vectors were the most efficient in transducing human DCs, with transduction efficiencies exceeding 95% at higher multiplicity of infection. Bronte *et al.* (1997) studied the antigen expression by DCs infected with a panel of rVVs in which a murine model tumor antigen was expressed under different promoters. Interestingly, DCs were found to express the model antigen only under the control of early promoters, even though late promoters were more active in other cell types. This study suggests that the use of promoters capable of driving the expression of TAA in DCs is essential in development of recombinant anticancer vaccines. Another

group recently described the effective generation of DCs expressing TAA by particle-mediated gene transfer (Tuting *et al.*, 1997). These DCs were able to induce antigen-specific CTLs *in vivo* and to reduce the growth of murine tumors expressing tumor-associated viral or "self" antigens. With our signal sequence method, we showed that human DCs can be loaded successfully with fusion peptides incorporating MART-1_{27-35} (Minev *et al.*, 1998). We found that the addition of signal sequence at the NH_2-terminus, but not at the COOH-terminus, of this epitope greatly prolonged its presentation in DCs. A newly designed peptide construct composed of the MART-1_{27-35} epitope replacing the hydrophobic part of a natural signal sequence was also effective. Interestingly, as with our earlier work with T2-cells, an artificial signal sequence containing the epitope was the most efficient construct for enhancing its presentation. These findings may be of practical significance for the development of synthetic anticancer vaccines and *in vitro* immunization of CTLs for adoptive immunotherapy. Although viral vectors are efficient vehicles for gene transfer into DCs, nonviral delivery of antigens has its advantages too. Fusion peptides can be readily produced in large quantities and are very stable. In addition, their application is not associated with immune responsiveness to vector-derived immunogens or with risk of recombination.

The encouraging results in experiments in mice, as well as improved techniques for *in vitro* immunization and expansion of DCs, support the initial attempts to immunize patients with DCs expressing tumor antigens. Development of an efficient method for isolation and partial purification of DCs (Takamizawa *et al.*, 1995; Mehta-Damani *et al.*, 1994) led to the infusion of antigen-pulsed DCs into four patients with follicular low-grade B-cell lymphoma (Hsu *et al.*, 1996). Complete remission was observed in two patients; one patient had a partial response and one patient had stable disease. In contrast, immunization with the antigen (monoclonal surface immunoglobulin) alone or emulsified in adjuvants did not induce regression of lymphoma (Kwak *et al.*, 1992). Another clinical study showed that DCs pulsed with idiotypic protein derived from serum in patients with multiple myeloma induced a specific CTL response in one patient (Reichardt *et al.*, 1996). DCs infected with poxviruses encoding MART-1 were able to sensitize T lymphocytes from melanoma patients *in vitro* (Kim *et al.*, 1997). This study suggests that the prolonged endogenous expression of TAA by the DCs might be utilized for induction of CTL responses in patients. However, the source of the DCs for vaccination and the frequency of the CTL precursors in cancer patients should be evaluated carefully. In patients with a low frequency of peptide-specific precursors, the efficient activation of antigen-specific CTLs required the use of peptide-loaded CD34+-derived, but not monocyte-derived, DCs (Mortarini *et al.*, 1997). This suggested that DCs derived from CD34+ cells and monocytes were not functionally equivalent for the activation of CTLs in patients with a low CTL precursor frequency. Antimelanoma CTLs were generated *in vitro* from healthy donors (Bakker *et al.*, 1995a) and melanoma patients (Storkus *et al.*, 1995) with DCs pulsed with melanoma-derived peptides. It was also shown that vaccination of patients with melanoma with DCs pulsed with MAGE-1-derived peptide elicited melanoma-specific CTLs *in vivo* (Mukherji *et al.*, 1995). In another clinical study, 16 melanoma patients were immunized with peptide-pulsed or tumor lysate-pulsed DCs (Nestle *et al.*, 1998). Vaccination was well tolerated in all patients. Objective clinical responses were observed in 5 of 16 patients with regression of metastases in various organs. These encouraging early clinical trials suggest that besides B-cell lymphoma, other cancers may also be responsive to DC-based immunotherapy.

3.5. *Heat Shock Proteins*

An interesting approach in vaccine development is the use of heat shock protein (HSP)-peptide complexes for vaccination. HSPs derived from any given cell type associate with a wide variety of peptides generated during protein degradation (Udono and Srivastava, 1993; Li, 1997). Vaccination of mice and rats with HSP-peptide complexes has resulted in powerful immune responses against the peptides bound to HSP, but not to HSP itself (Udono *et al.*, 1994; Udono and Srivastava, 1993, 1994). A disadvantage of this approach is the requirement for generation of customized, patient-specific vaccines for cancer. However, an attractive feature of the vaccinations with HSP-peptide complexes is their ability to elicit a specific CTL response in mice of any haplotype. This may be because the association of peptides with HSP occurs before their association with the MHC molecules, and, therefore, these peptides are not associated with any particular haplotype (Suto and Srivastava, 1995; Srivastava *et al.*, 1994).

3.6. *Adjuvants*

Vaccines derived from tumor cells or tumor lysates were used before the identification of TAA (Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell, 1988). Various adjuvants were important to improve the efficiency of these vaccines in eliciting polyvalent antitumor immune responses. Since a main disadvantage of peptide-based vaccines is their low immunogenicity, careful selection of the adjuvants used with these vaccines is even more important. The function of each adjuvant depends on its ability to affect the pathway of the antigen presentation, to prolong the antigen exposure to the APCs, as well as to influence the number and the type of the APCs and the release of cytokines in the local environment. New adjuvants currently being developed include preparations that resemble IFA, preparations derived from bacterial cell wall and modified in order to reduce toxicity, and different cytokines (McElrath, 1995). Use of cytokines as vaccine adjuvants is very attractive since it may allow precise modulation of the direction of the immune response (Biron and Gazzinelli, 1995; Dranoff *et al.*, 1993). Since granulocyte/macrophage colony-stimulating factor plays an important role in the maturation and function of APCs

such as DCs and macrophages (Reid *et al.*, 1992), it has attracted attention as a cytokine adjuvant (Disis *et al.*, 1996; Dranoff *et al.*, 1993). The use of other cytokines, such as IL-2, IL-12, and IL-10, as vaccine adjuvants was reported. The efficacy of recombinant poxvirus-based vaccines was greatly improved by the addition of exogenous IL-2 to the vaccination regimen (Bronte *et al.*, 1995). IL-12 is also a promising vaccine adjuvant because of its role in directing Th1 responses and thus, CTL responses (Rao *et al.*, 1996; Meko *et al.*, 1995; Biron and Gazzinelli, 1995). These studies suggest that the cytokines and other adjuvants are an important component of the optimal vaccine formulations.

4. PROSPECTS

The antigenic profile of human tumors is very complex and consists of many peptides originating from various classes of protein. This fact should be considered carefully in designing anticancer vaccines. An important question is which tumor antigens are the most important in tumor regression *in vivo*. Differentiation antigens may play an important role in tumor regression, which is suggested by the positive correlation between the development of vitiligo and a good clinical response to immunotherapy in melanoma patients (Nordlund *et al.*, 1983). Promising candidates are also MAGE, BAGE, and GAGE antigens since they are expressed in a variety of cancer cells, but not in normal cells except testis. Mutated epitopes, such as CDK-4 and β-catenin, are tumor specific, but immunotherapy using these antigens is likely to be applicable only to individual patients. In any case, the ideal vaccine most likely will consist of a cocktail of tumor antigens or proteins. However, the number of epitopes in the vaccine cocktail should be evaluated carefully since CTL responses directed to fewer epitopes in patients with acquired immunodeficiency syndrome are associated with a better clinical outcome (Nowak *et al.*, 1995). In this case, it appears that the stimulation of multiple simultaneous CTL responses is clinically inefficient. Very important also is the dose of antigen and the speed of antigen release in the vaccine formulations. High doses of antigen released faster may induce T-cell tolerance (Toes *et al.*, 1996). Immune tolerance may be due to fast expansion and subsequent elimination of specific T-cell clones, or to apoptosis induced by repeated stimulation of already stimulated T-cells in cell cycle (Webb *et al.*, 1990; Aichele *et al.*, 1995). Therefore, it is essential to select as immunogens those epitopes against which tolerance has not been induced (Benichou *et al.*, 1994; Sercarz *et al.*, 1993).

One must not overlook the use of whole tumor cells or membrane preparations expressing all the antigens of interest. Our current understanding of the function of the costimulatory molecules might lead to the development of more efficient modified whole cell vaccines. The use of the entire antigenic proteins might well be superior to peptide vaccines. A whole protein may provide several T-cell epitopes presented by different MHC Class I molecules, which is the case with tyrosinase (presented by HLA-A2 and HLA-A24), MAGE-1 (presented by HLA-A1 and HLA-Cw16), and MAGE-3 (presented by HLA-A1 and HLA-A2). An additional advantage of the whole protein vaccines may be the induction of humoral immune responses (Hoon *et al.*, 1995).

In conclusion, it is clear that many challenges exist in the design of the optimal anticancer vaccines. However, a new enthusiasm in this exciting field is supported by the rapid advances in our understanding of the basic mechanisms of antigen presentation, as well as by the promising initial clinical studies. This enthusiasm undoubtedly will lead to the development of more effective and specific anticancer vaccines in the future.

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EXHIBIT B

A Phase I/II Randomized Study of

Flt 3 Ligand (FL)

with or without vaccination in HLA-A2+ patients

with Stage II, III and IV malignant melanoma

LICR Protocol No: 97- 012

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	Amendment 5	28 Jun 1999
	Amendment 6	04 Nov 1999
	Amendment 7	10 Sep 2000

PROTOCOL SYNOPSIS

Title
A Phase I/II Randomized Study of Flt 3 Ligand (FL) with or without vaccination in HLA-A2+ patients with stage II, III and IV malignant melanoma.

Study Phase
Phase I/II

Objectives
Primary Objectives
1 To describe the safety and toxicity of FL alone and in combination with NY-ESO-1 peptides, tyrosinase peptide, Melan-A peptide and influenza peptide.

2 To describe the safety of FL in combination with imiquimod and in combination with NY-ESO-1 peptides, tyrosinase peptide, Melan-A peptide and influenza peptide.

Secondary Objectives
1 To determine whether there is any preliminary evidence that FL induces immune responses to NY-ESO-1, tyrosinase, Melan-A or Influenza antigens with or without vaccination with peptides derived from these proteins.

2 To document tumour responses in patients treated with FL alone, or combined with NY-ESO-1 peptides, tyrosinase peptide, Melan-A peptide and influenza peptide.

3 To determine whether the application of imiquimod to the skin at vaccination sites enhances any immune or clinical effects.

Study Design
This is a two arm randomized phase I/II study designed to describe the toxicity response, immune response and tumour responses of patients with malignant melanoma to treatment with FL alone, or FL combined with melanoma antigen peptides. The sole purpose of randomization is to minimize the impact of random imbalances in prognostic variables. The study is not intended to provide an adequately powered comparison between arms. A further arm has been added (Amendment 7) to evaluate the effect of imiquimod cream at vaccine sites. Randomisation will not be performed for these patients.

Patients
24 HLA A2+ Patients
Tumour Type: Malignant Melanoma
Patients will have stage II, III or IV malignant melanoma with an expected survival of greater than 4 months.
Tumours should test positive for either NY-ESO-1, MelanA or tyrosinase by immunohistochemistry or RT-PCR.

Investigational Agents
1 FL, solution, S/C, (Immunex Corporation)
2 NY-ESO-1 peptide (SLLMWITQC, SLLMWITQCFL and QLSLLMWIT) solution, ID, (Multiple Peptide Systems, San Diego)
3 Influenza matrix peptide (GILGFVFTL) solution, ID, (Multiple Peptide Systems, San Diego)
4 Tyrosinase internal sequence peptide (YMDGTMSQV) solution, ID, (Multiple Peptide Systems, San Diego)
5 Melan A peptide (ELAGIGILTV) solution, ID, (Multiple Peptide Systems, San Diego)
6 Imiquimod cream (Aldara cream 5%), 3M Pharmaceuticals, St Paul, Minnesota, USA

Special Procedures

1 Assessment of cellular immune responses.
   - DTH reactions to: tetanus toxoid, diphtheria, streptococcus, tuberculin, Candida, Trichophyton, Proteus mirabilis, and a glycerol control.
   - Ag-specific T cell responses to peptide epitopes of specific (vaccinated) and other (non-vaccinated) antigens pulsed onto T2 cells or autologous irradiated peripheral blood mononuclear cells (PBMC) and HLA-A2+ melanoma cell lines by chromium release.
   - Induction of cytokine expression/secretion by responder T lymphocytes in peptide presentation assays using ELISpot assays.
2 Assessment of humoral immune responses to peptide epitopes by ELISA.
3 Antigen-presenting cell studies.
   - Skin: Biopsy to define infiltrating mononuclear cells and Langerhans cells
   - Blood: Detection of circulating peripheral blood monocytes and DC by multi parameter flow cytometry (FACS). Phenotypic and functional analysis of FL-generated DC subsets including receptor mediated endocytosis or phagocytic capacity respectively.

Study Endpoints

- Safety endpoints will include toxicity and serious adverse events
- Immune response endpoints will include T-cell cytotoxicity, T-cell release of Interferon-gamma by ELISpot, DTH reactions and the numbers of dendritic cells in blood
- DC endpoints include changes in the number and function of dendritic cells in blood and skin
- Tumour response endpoints will include changes in tumour size and the disappearance or appearance of tumours

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Statistical Considerations

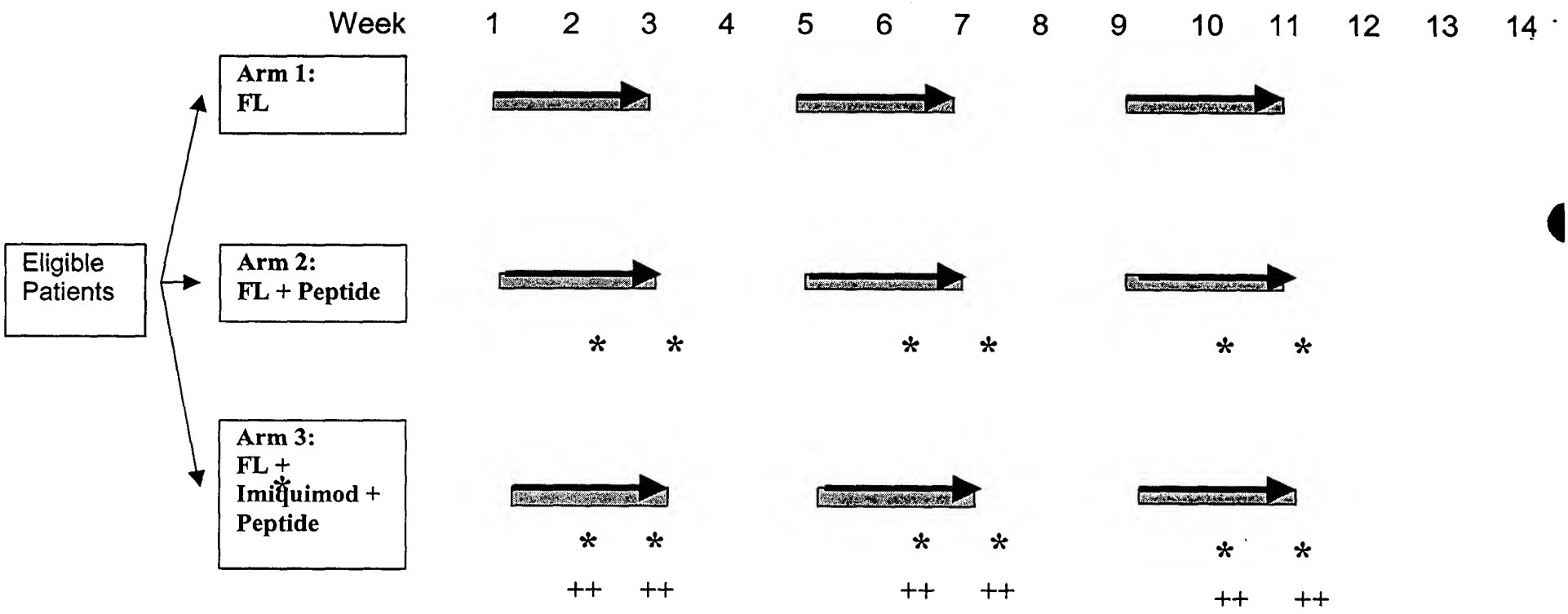
Eight patients will be studied in each arm. This will be sufficient to describe the safety, toxicity, immune effects and any clinical response profiles for each therapy.

This study design will therefore establish whether or not there is toxicity or activity (clinical and/or immunological) attributable to each regimen. The numbers in this study will not be sufficient to perform statistically significant comparisons between each arm.

Sponsor

Ludwig Institute for Cancer Research

Protocol Schema: A Phase I/II Randomized Dose Finding Study of Flt 3 Ligand (FL) with or without vaccination in HLA-A2+ patients high-risk stage II, III and IV malignant melanoma.



FL 20ug/kg/day (maximum dose 1.5mg (1 vial)/day)

*Peptides: SLLMWITQC, SLLMWITQCFL, QLSLLMWIT, GILGFVFTL, YMDGTMSQV, ELAGIGILTV (Multiple Peptide Systems) ID on D8 & 15 of each cycle

+Imiquimod cream: will be applied twice to skin underlying peptide vaccination sites 24 hours before vaccination and 2 hours before vaccination.

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1.0 OBJECTIVES

Primary Objectives

1 To describe the safety and toxicity of FL alone and in combination with NY-ESO-1 peptides, tyrosinase peptide, Melan-A peptide and influenza peptide.

2 To describe the safety of FL in combination with imiquimod and in combination with NY-ESO-1 peptides, tyrosinase peptide, Melan-A peptide and influenza peptide.

Secondary Objectives

1 To determine whether there is any preliminary evidence that FL induces immune responses to NY-ESO-1, Tyrosinase, Melan-A or Influenza antigens with or without vaccination with peptides derived from these proteins.

2 To document tumour responses in patients treated with FL alone, or combined with NY-ESO-1 peptides, Tyrosinase peptide, Melan-A peptide and influenza peptide.

3 To determine whether the application of imiquimod to skin at vaccination sites enhances any immune or clinical effects.

2.0 BACKGROUND AND RATIONALE

2.1 Disease

Patients with stage II melanoma have a 5 year survival of 75%, stage III have a 5 year survival of 40% and stage IV melanoma have a median survival of less then one year and a less-than 5% chance of long-term survival (Balch 1992). Although treatment with dacarbazine or drug combinations including dacarbazine has been considered standard therapy, response rates range only from 8-25% and there is no evidence that dacarbazine or any other treatment extends survival. Patients with high risk stage III melanoma have a median survival of one to two years and a low chance of long-term survival. New approaches to therapy are needed.

2.2 Investigational Agents

Flt3 Ligand (FL)

Flt3 is a tyrosine kinase growth factor receptor. The ligand (FLT-3L, FL) has structural similarities with steel factor (Stem cell factor (SCF)) although each factor probably has a unique role in haematopoiesis, primarily on primitive progenitor and dendritic cells. Unlike SCF, it appears to have no effect on mast cells. Flt3 expression can be detected on a wide variety of tissues including primitive haematopoietic cells and malignant myeloid and lymphoid cells (Lyman 1994)

Effects on Haematopoietic Progenitors
Flt3 has activity on progenitors of multiple lineages including GM-CFC, G-CFC, GEMM -CFC, Meg-CFC, BFU-E and lymphoid progenitors. In vitro, it can amplify primitive long-term culture-initiating cells LTC-IC (Petzer 1996). It can act on primitive/quiescent CD34+/38- cells and recruit these into cycle (Haylock 1997). This may explain the potent synergy observed in vitro and in vivo when FL is combined with G-CSF, GM-CSF, IL-3 and IL-6.

Progenitor Mobilization
FL is potent at mobilizing haematopoietic progenitors in mice including primitive progenitors such as CFU-S (Ashihara 1998) Following in vivo administration marrow, spleen and peripheral blood colony forming cells were expanded. This was particularly evident when FL was combined with G-CSF or GM-

CSF and peripheral blood CFC mobilization was measured (Brasel 1997). In Scid-hu mice bearing human marrow the effects of FL on human progenitor mobilization was accentuated by prior irradiation, an effect which might be explained by the release of synergizing cytokines as a result of radiation/ myelosuppression (Namikawa 1999).

Haematopoietic Effects In Primates

Recombinant human FL was administered to non-human primates for 12 or 14 days. It was found to mobilize early haematopoietic progenitor cells (GM-CFC, BFU-E, GEMM-CFC, lymphoid progenitors) (Papayannopoulou 1997) and expand bone marrow progenitors and cellularity (Winton 1995.). The only observed clinical effects were mild lymphadenopathy and splenomegaly. Monocytes were increased 11-fold (Winton 1995.).

Effects on Dendritic Cells

In mice, FL administered daily for 9 days was seen to dramatically expand dendritic cells (DC) in spleen, peripheral blood, lymph nodes, bone marrow, peritoneum, lung and liver. DC increased after 2 days of FL administration and by day 9 cells appeared to be phenotypically mature DC with increased surface expression of MHC Class II, CD11c, CD86 and DEC205. In vivo, FL was more potent than GM-CSF at increasing total numbers of splenic DC (Maraskovsky 1996). These DC were potent in presenting allo-antigen or soluble antigen to T cells, or in priming an Ag-specific T cell response in vivo (Maraskovsky 1997).

DC pulsed with tumour antigens ex vivo and re-infused into animals have been shown to generate an effective T cell-mediated, anti-tumour immune response in several models of established tumours. The immunological consequences of increased DC numbers in FL-treated animals is currently being investigated, however, there is now preliminary evidence that FL can stimulate immune responses which result in the regression and rejection of murine tumours (Chen 1997a; Lynch 1997). The mechanisms for the anti-tumour effects of FL appear to be due to the generation of a T cell-mediated anti-tumour immune response as well as NK-mediated anti-tumour effects (Lynch 1997).

In a randomized, double blind, placebo-controlled clinical study, normal healthy volunteers received FL subcutaneously for 14 consecutive days at doses of 10, 25, 50, 75 or 100 ug/Kg/day (Maraskovsky 2000). Elevated WBC counts with increased monocytes were consistently observed. Consistent with the mouse studies, circulating DC were increased 50-fold in FL-treated individuals as assessed by flow cytometric analysis. These DC appeared to be phenotypically and functionally immature 'processing-type' DC, which are the most appropriate type of DC for the effective capture of vaccine Ag. Furthermore, all events related to FL administration (including the expansion in circulating DC) were reversible upon discontinuation of FL. No serious adverse effects were observed.

Side effects Associated with FL

In the normal volunteer study FL was well tolerated, with transient, mild increases in LDH, SGOT and SGPT observed in some patients whilst the only two adverse events recorded were mild injection site reactions and enlarged lymph nodes which were thought to be associated with FL treatment. No allergic type reactions or other signs of mast cell degranulation were observed.

Leukocytosis is an expected pharmacological effect of FL. Studies in mice and monkeys showed dose dependent effects of higher doses of FL (elevated WBC counts, anemia, lymphadenopathy, hepato-splenomegaly). Mononuclear cell tissue infiltrates, observed in monkeys following 30 days of FL administration, were reversible with discontinuation of drug. Rare foci of hepatic necrosis, observed in toxicology studies, were attributed to infiltrates resulting from mobilization of haematopoietic cells and were accompanied by increases in serum transaminases. Although there is no evidence that healthy animals develop autoimmune disease following FL administration, it is unknown whether FL may exacerbate autoimmune disorders by its immunomodulatory effects. Therefore, subjects at increased risk for or with a personal history of autoimmune disease should not receive FL. In humans FL has been generally safe and well tolerated at the doses tested (10-100 µg/kg/day for up to 14 days). Elevated WBC counts with increased monocytes were universal. Lymphadenopathy, erythema and swelling at the injection sites, mild increases in LDH, AST, and ALT and decreases in platelet counts have been observed. All events were reversible with discontinuation of FL administration. Differential WBC counts

and serum chemistries should be monitored in subjects receiving FL. When FL has been combined with other molecules (G-CSF or GM-CSF) additional side effects have been reported. These include constipation, protein in the urine, vomiting, red eyes, dizziness, increased blood clotting and sweats. These events may or may not have been related to FL. A number of drugs used to treat cancer can cause secondary cancers or leukaemia. FL is not known to be associated with this risk and we have no reason to believe that this will occur, nonetheless we cannot exclude this possibility.

Additionally, with FL there have been infrequent reports of back and muscle pain, malaise (tiredness), nausea, diarrhea, pharyngitis, headache, herpes simplex, dyspepsia, and haematuria. These events may or may not have been related to the drug.

The risks to a fetus (unborn child) in this study are unknown; therefore, any person and/or their partner on this study must use an accepted and effective method of contraception throughout the study.

In the present protocol, we propose to treat patients with evaluable stage 3 or 4 malignant melanoma subcutaneously with FL at 20 ug/Kg/day. In addition, we propose to also treat separate cohorts of these melanoma patients with FL in combination with a mixture of tumour Ag peptides.

Imiquimod [1-(2-methylpropyl)-1H-imidazol[4,5c] quinolin-4-amine]

Imiquimod is a topical cream which has been used for the treatment of external genital and perianal warts. (Edwards 1998). It is an immuno-modulating agent which is believed to act by inducing cytokines. Cytokines which it has been shown to induce include interferon (IFN)-α, tumour necrosis factor (TNF)-α, interleukin (IL)-1α, IL-6, and IL-8 (Sidky 1992; Witt 1993; Kono 1994; Reiter 1994; Weeks 1994; Gibson 1995; Testerman 1995). Although it does not appear to have direct antiviral activity, induction of these cytokines has demonstrated efficacy against viral infections and some tumours. These include anogenital warts, basal cell carcinoma(Beutner 1999) and cutaneous melanoma metastases(Steinmann 2000). The main reported adverse events relate to local skin reactions which are reported as being severe in fewer than 5% of patients. Erythema has been reported in 3% of patients, ulceration in 2% and oedema in 1%. Reactions were most frequent with daily application. In this study, imiquimod will only be applied twice. Imiquimod is approved in Australia for the treatment of anogenital warts. Toxicity in conjunction with FLT-3 ligand is not known.

A recent study demonstrated that imiquimod induces migration of Langerhans cells from skin to regional lymph nodes (Suzuki 2000). This migration was associated with the induction of cytokines. These studies provide the basis for using imiquimod as a 'maturation' stimulus in the current protocol.

Tumour Antigens

A number of tumour antigens have recently been characterized and have enabled clinical trials to commence using defined antigens. This represents a major advance over previous clinical trials which used autologous irradiated cells, transfected cell lines and cell lysates or supernatants. These systems, which used poorly defined antigens, were often difficult to evaluate because specific immunological responses were not easily monitored. By using defined antigens as targets it has been possible to develop specific immunological tests based on these targets, making it possible to better monitor trials using immunological endpoints. In addition, defined antigens enable standardization and quality control of clinical reagents.

Defined tumour antigens which could be considered for clinical cancer vaccines include a recently defined group of antigens designated "CT antigens" (Cancer/Testis antigens). These antigens have a number of features in common. They are frequently expressed in cancers but only a very limited range of normal tissues (Testis, ovary). Antigens in this family include MAGE (a family of proteins consisting of twelve homologous members); BAGE; GAGE (Reviewed (Van den Eynde 1997)) and the more recently defined antigens MEL 40 (Sahin 1995) and NY-ESO-1 (Chen 1997b). Common features of this group of antigens include a limited pattern of tissue expression, chromosomal localization on the X chromosome, and expression in between 20 and 50% of human cancers. Expression appears to be

more commonly observed in the later stages of tumour development, such as in metastatic lesions. For the MAGE antigens, this appears to be due to demethylation of the MAGE promoter.

In melanoma, differentiation antigens including tyrosinase, Melan A and gp100 have been identified as potential targets for immune rejection. These are described in greater detail below.

These antigens were initially defined using cultures of human melanoma cells and autologous lymphocytes. Cytotoxic T-cells (CTL) could be generated that lyse the autologous tumour cells but do not lyse autologous fibroblasts or EBV transformed B-lymphocytes, or target cells susceptible to lysis by natural killer cells. The identification of genes encoding tumour antigens recognized by CTL has made it possible to select patients whose tumour expresses the antigens and who could therefore be eligible for immunization.

NY-ESO-1:

NY-ESO-1 is an antigen identified following SEREX analysis from a patient with esophageal squamous cell carcinoma. The pattern of mRNA expression in normal tissues was restricted, with high-level mRNA expression found only in testis and ovary tissues. Reverse transcriptase-PCR analysis showed NY-ESO-1 mRNA expression in a variable proportion of a wide array of human cancers, including melanoma, breast cancer, bladder cancer, prostate cancer, and hepatocellular carcinoma. NY-ESO-1 encodes a putative protein of Mr 17,995 having no homology with any known protein. The pattern of NY-ESO-1 expression indicates that it is also a CT antigen (Chen 1996, Chen 1997b). Recently, three overlapping HLA A2-restricted epitopes for NY-ESO-1 have been described (Jager 1998). NY-ESO-1 peptides have not previously been administered to humans. Any potential adverse events cannot be predicted, nonetheless, for the other melanoma associated antigens, administered intradermally, peptide injections were associated with little or no toxicity. The ESO peptides will be diluted to form a solution containing 33% DMSO in saline solution.

Melanocyte differentiation Antigens:

These are derived from proteins which are associated with melanocyte lineage cells. HLA restricted peptide antigens have been identified from the enzyme tyrosinase (Brichard 1993; Wölfel 1994), an enzyme which plays a role in the melanin synthesis. The proteins Melan A (Coulie 1994) and gp100(Kawakami 1994) have also been identified and as peptides are being tested in early phase clinical studies (Davis 1999). Different studies are currently conducted using peptides for immunization that are derived from differentiation antigens of the melanocyte lineage and are presented by the HLA-A2 molecule of melanoma cells and normal melanocytes. Peptides derived from Melan A/MART-1, tyrosinase, and gp100/Pmel17 have been used for immunization (Davis 1999). Intradermal injections of the tyrosinase derived peptides peptide have elicited DTH reactions in some of the vaccinated patients. Toxic side effects were not observed. The generation of peptide-specific CTL responses could be assessed after completion of the vaccination schedule in some of the immunized patients. Minor regression of metastatic lesions was observed in some of the patients who had developed CTL responses to the vaccine. CTL responses have been assessed using a 'Mixed Lymphocyte Peptide Culture' (MLPC) assay. Baseline peptide-specific CTL reactivity was determined in the peripheral blood of melanoma patients and compared to peptide-specific CTL reactivity after intradermal immunization with melanoma associated peptides (Jager 1996a; Jager 1996b).

To date no serious adverse effects attributable to the Melan A, tyrosinase or flu peptides have been observed (Cebon et al, unpublished observations, Lienard 1997, personal communication) or reported (Jager 1996b), In some cases local inflammatory responses have been observed at the sites of intradermal injection (Lienard 1997, personal communication). In the case of the flu peptide these reactions were up to 1 cm in diameter and associated with mild vesiculation. In all cases these were self limiting reactions. By inference, we do not anticipate significant toxicity with the NY-ESO-1 peptides. Nonetheless, patients will be monitored closely for any adverse effects.

2.3 Rationale

Preliminary results suggest that FL may be an important cytokine for the expansion of large numbers of functionally mature DC in vivo for use in clinical immunotherapy either as a single agent or as a cytokine adjuvant to enhance immune rejection of tumours following vaccination with tumour antigens. In this way, FL administration maybe an effective means to actively immunize patients without the need for culturing DC ex vivo.

The purpose of this study is to determine whether FL, administered at a dose of 20 μg/Kg/day is tolerable, influences DC and DC precursor expansion and has immunological efficacy when administered with or without defined antigens.

FL has been shown to be generally safe, well tolerated and biologically active at the doses tested (10-100 μg/Kg/day for up to 14 days). The dose of 20 μg/Kg/day was chosen because it is in the dose range shown to be biologically active with respect to both DC expansion and progenitor mobilization in phase I studies in normal volunteers (Maraskovsky 2000)

The endpoints of this study will be predominately immunological and will be based on responses to several antigens: NY-ESO-1 peptides (SLLMWITQC, SLLMWITQCFL and QLSLLMWIT), Influenza Matrix peptide GILGFVFTL, the Melan A peptide (ELAGIGILTV) and Tyrosinase internal sequence peptide (YMDGTMSQV).

Reactivity against these antigens in non-vaccinated subjects who receive FL alone will provide valuable information about the effects of FL on CTL populations in the absence of specific immunization. Correlation with any clinical responses will be sought. We will also screen for either reactivity against the EBV LMP-2 peptide CLGGLLTMV (residues 426-434)(van der Burg et al 1996) and the Melan A 9-mer peptide AAGIGILTV (residues 27-35). It is expected that some patients will have pre-existing reactivity against these epitopes. Such pre-existing CTL responses can therefore be followed prospectively during FL administration.

3.0 EXPERIMENTAL PLAN

3.1 Study Design

Study Phase: Randomised Phase I/II, open-label, three-arm study.
A total of 24 patients will be treated.

- FL administered alone at 20 μg/kg/d for 14 days on weeks 1&2, 5&6 and 9&10
- FL administered at 20 μg/kg/d for 14 days on weeks 1&2, 5&6 and 9&10 plus peptide antigen vaccination on days 8, 15, 36, 43, 64 and 71
- A third arm will be added for the next 8 patients:
  FL administered at 20 μg/kg/d for 14 days on weeks 1&2, 5&6 and 8&99&10 plus peptide antigen vaccination on days 8, 15, 36, 43, 64 and 71. Imiquimod will be applied to skin at peptide vaccine sites prior to peptide injection on days 7+8, 14+15, 35+36, 42+43, 63+64 and 70+71.

Each arm involves 3 cycles of treatment at 4 weekly intervals. See schedule of tests.

Study agents will be administered according to the schema on page vi. FL will be administered for 2 weeks at 4 weekly intervals. Vaccination will be performed on two occasions (1 week apart) during each cycle. Patients will have peripheral blood collected for standard hematology and biochemistry as well as studies of dendritic cell populations and cytotoxic lymphocytes reactive with specific antigens.

Leukapheresis will be performed prior to commencement on study and at the completion of vaccination and FL treatment on day 85. These procedures are performed in order to obtain sufficient lymphocytes for detailed immunological studies.

A total of 24 patients will be treated. Previous studies have used FL in doses up to 100 μg/Kg/day. Lower doses have been shown to exert similar biological effects and 20 μg/Kg/day will be used in this study.

After screening and registration, patients will be randomized to receive one of the two regimens. Patients 17-24 will receive imiquimod in addition to FL and peptides.

Study endpoints relate to safety, immunology and tumour responses. These are detailed in section 10.2

3.2 Number of Institutions

The study will be performed at the Ludwig Institute Oncology unit at Austin & Repatriation Medical Centre.

3.3 Number of Patients

24 patients will be recruited, 8 in each arm.
1 FL alone (14 day cycle),
2 FL (14 day cycle)+ antigens
3 FL (14 day cycle) + imiquimod + antigens.

Patients who discontinue will be replaced unless this results from undue toxicity. Stopping rules are listed in section 6.3.

3.4 Estimated Study Duration

Estimated study duration is 18 months. 2-3 patients are planned to be entered each month. Patients will discontinue the treatment phase after a final assessment on week 15.

Observation Phase. Patients will continue to be followed according to the schedule of tests at 6 and 12 months. This is to monitor for longer term autoimmune effects.

4.0 PATIENT ELIGIBILITY

4.1 Inclusion Criteria

Patients must have stage II, III or IV malignant melanoma or in-transit metastatic disease, confirmed by review of histology.

Patients must have fully recovered from surgery.

Patients must have an expected survival of at least four months.

Histological review: Tumours should be positive by immunohistochemistry or RT-PCR for at least one of the melanoma antigens: tyrosinase, MelanA, NY-ESO-1

Performance status: Karnofsky Performance Status of 70% or better.

Patients must have the following results of laboratory studies: serum creatinine of < 0.2 mmol/L, bilirubin < 25 umol/L, serum AST/ALT less than two times the upper limit of normal, unless due to the presence of hepatic metastases, WBC >3.0 x 10^9/L granulocytes > 2.5 x 10^9/L, lymphocytes > 0.5 x 10^9/L, platelets > 100 x 10^9/L.

Patients will be HLA tissue type A2 +ve.

Patients must be age 18 to 75 years.

Before any trial-specific procedures, randomization or treatment can be performed, the patient or legally authorized guardian or representative must give witnessed written informed consent for participation in the trial (see Section 12.2)

4.2 Exclusion Criteria

Clinically significant heart disease - NYHA Class III or IV. (Appendix A);

Other serious illnesses, e.g. active infections requiring antibiotics, bleeding disorders;

Other contraindications to leukapheresis;

Patients cannot have other types of cancer that coexist or that have been previously treated apart from in situ carcinoma of the cervix or basal cell carcinoma of the skin;

Metastatic disease to the central nervous system; Patients with previous, treated disease involving the CNS may be included if disease has been treated by surgery and or radiotherapy and there is no evidence or disease progression in the CNS either radiologically or clinically for three months or more;

Four weeks must elapse after previous radiation therapy, chemotherapy (6 weeks after nitrosourea drugs) or immunotherapy before the patient can be entered;

Known positivity for HIV;

A history of autoimmune disease, excluding vitiligo and diabetes mellitus;

Previous splenectomy or radiation therapy to the spleen;

Concomitant drug treatment with systemic corticosteroids, non-steroidal anti-inflammatory drugs or other immunosuppressive therapy;

Patients may not receive other investigational agents at the same time;

Pregnancy or lactation. Women of child-bearing potential must have a negative pregnancy test at the time of entry. Patients need to practice effective contraception at entry and during participation in the trial;

Psychiatric, addictive or other disorders that compromise the ability to give informed consent;

Patients who are not geographically accessible for evaluation or follow-up assessment.

4.3 Concomitant Medications and Treatments (ancillary therapy)

The following medications and treatments should not be administered within two weeks of entry into the trial or during participation in the study: systemic corticosteroids, NSAIDs, unless clinically indicated for treatment of an acute medical episode and there is no reasonable alternative effective therapy.

5.0 PATIENT ENROLMENT

Before entering patients into the trial, written Institutional Review Board approval of the protocol and informed consent form must be obtained (see Sections 12.3, 12.2, respectively).

5.1 Treatment Assignment

Patients will be assigned successive numbers on registration into the study.

5.2 Randomization

Treatment Assignment: The first 16 patients will be assigned to the treatment arms on the basis of a randomization schedule which is located in the Pharmacy department, ARMC. For randomization of relevant patients contact the Clinical Trials Pharmacist on 9496 3178. Patient numbers will be assigned sequentially on the basis of accrual to the study (time when informed consent is given). If there are circumstances when patients may be replaced, refer to section 8.4 for details.

Patients 17-24 will not be randomized.

6.0 TREATMENT PLAN

6.1 Agent Doses

FL is administered daily for 14 days, subcutaneously at a dose of 20 μg per kilogram per day.

Peptides will be administered by ID injection twice each cycle on days 8 and 15, 36, 43, 64 and 71. Peptide dose 100 ug (influenza matrix peptide GILGFVFTL, Melan A peptide ELAGIGILTV and tyrosinase YMDGTMSQV) for the NY-ESO-1 peptides (SLLMWITQC, SLLMWITQCFL and QLSLLMWIT) the dose is 33 ug.

Imiquimod 750 mg (3 sachets) will be applied one day prior to peptide injection and on the day of peptide injection.

6.2 Agent Administration and Schedule

FL will be administered by daily subcutaneous injection for 14 days. The FL injection site should be rotated at sites which are distant from the site of tumour antigen vaccination.

Imiquimod (Aldara cream 5%) will be applied to skin of the vaccine 24 hours and 2 hours prior to each peptide vaccination. One sachet (250mg) is sufficient to cover 20 cm^2 of skin. One sachet will be sufficient for two vaccine sites. A total of 3 sachets will be required for each day of treatment: approximately 10 cm^2 of skin should be treated at each vaccine site.

Sites of injection will preferably be on the limbs. Each peptide injection must be injected intradermally to give a visible and palpable depot. Injection sites should be 10cm or more apart. The location of each ID injection should be clearly marked for later evaluation of DTH reaction. Avoid using limbs which have been subject to regional lymphadenectomy or radiotherapy to draining nodes. All treatments may be administered on an out-patient basis. Refer to protocol schema.

Each ESO peptide (provided at 100 μg in 330 μL) will be diluted 1 in 3 using sterile saline solution. A total of 330 micro litres (33 μg) will be injected.

Further vaccination with peptides alone can be offered to patients, at the discretion of the investigator.

6.3 Dose Adjustments

Any CTC grade 3 or 4 toxicity attributable to study agent will require cessation of FL until the toxicity resolves.

If Grade 1 or 2 toxicity attributable to FL occurs in an individual patient, FL will be continued at full dose. If Grade 3 non-major organ toxicity attributable to FL occurs, FL will be interrupted for that cycle and restarted in the next cycle if the toxicity has resolved ≤ Grade 2. If Grade 3 or 4 major organ toxicity attributable to FL occurs in an individual patient, including autoimmune reactions, FL will be discontinued in that patient.

Dose adjustment of vaccine antigens is not required. Vaccination may continue without FL once toxicity has resolved to grade 2 or better by the next cycle. If severe (grade 3 or 4) hypersensitivity to a defined antigen occurs, vaccination with that particular antigen should not be continued.

If any delay of study procedures occurs as a result of adverse events, and this delay exceeds 30 days, the patient will be removed from study. For rules covering replacement of patient see Section 8.4.

In arm 3, grade 3 skin toxicity at the site of peptide injection may require discontinuation of imiquimod. This will be at the discretion of the Principal Investigator and will be evaluated on a case-by-case basis.

6.4 Maximum Tolerated Dose (MTD)

Not applicable to this protocol.

6.5 Dose-Limiting Toxicities (DLT)

Not applicable to this protocol.

6.6 Dose-Escalation Criteria

Not applicable to this protocol.

6.7 Ancillary Therapy

Concomitant medications to control side-effects of therapy will be given if patients have documented fevers and chills at any stage during treatment. Pethidine 25 mg, 3 hourly may be given intravenously if rigors develop. Other supportive therapy will be given as required. Patients should not be administered steroids. All prescription and nonprescription concomitant medications must be recorded in the case report form, listing generic name, indication, quantity administered, and dates of administration.

6.8 Other Treatment Procedures

As per schedule of tests.

- Blood Tests
- Scans and X-Rays
- Immunological Studies
- Skin biopsies
- Biopsies of peptide vaccination sites
- Leukapheresis

6.9 Schedule of Tests

	Pre	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13
		MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS	MTWTFSS
Study day		1	8	15	22	29	36	43	50	57	64	71	78	85
Arm 1														
FL		XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			
Arm 2														
FL		XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			
Vaccine		XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Arm 3														
FL		XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			XXXXXXX	XXXXXXX			
Vaccine		XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Imiquimod		XXXXXXX	XXXXXXXX	XXXXXXXX		XXXXXXXX	XXXXXXX	XXXXXXX		XXXXXXX	XXXXXXX	XXXXXXX		
History	x	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Clinical Examination ++	X*	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	X*XXXXX
Clinical Examination ++	X*	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	X*XXXXX
Imaging #	x	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Screening tests	A	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
Toxicity Evaluation	B	XXXXXXX	XXXXXXX	BXXXXXX	XXXXXXX	BXXXXXX	XXXXXXX	CXXXXXX	XXXXXXX	BXXXXXX	XXXXXXX	BXXXXXX	XXXXXXX	CXXXXXX
Immunology tests	(G)	XXXXXXX	XXXXXXX	XXXXXXX	DXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	DXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXX	(G)XXXXX
DC studies	(G)	XXXXXXX	EXXXXXX	FXXXXXX	XXXXXXX	EXXXXXX	EXXXXXX	FXXXXXX	XXXXXXX	EXXXXXX	EXXXXXX	FXXXXXX	XXXXXXX	EXXXXXX
Leukapheresis	G	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	GXXXXXX
Blood Volume	100		10	165	150	25	10	175	150	25	10	165	0	85
Inflam. Response Blood Volume✣	6		16	6	16	6	0	6 (opt.)		6 (opt.)		6 (opt.)		6 (opt.)
Cumulative Blood Volume	106		132	303	469	500	510	691	841	872	882	1053	1053	1144
Skin biopsy for DCs (4 mm)	X	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX
DTH Biopsies (Arm 2)		XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX	XXXXXXX

Schedule of Tests: Follow on phase:

	15 weeks (off study)	6 months	12 months
History	X	X	X
Clinical Examination*, ++	X	X	X
Imaging to stage disease#	X		
Toxicity Evaluation	B	B	B
Immunology Laboratory tests			D

Legend for assessment schedule

A: **Screening tests:** Histological review and tumour typing by immunohistochemistry or RT-PCR for tyrosinase, MelanA, NY-ESO-1, Full blood examination including differential white cell and platelet count, PT/PTT, Blood chemistry: serum urea, creatinine and electrolytes, serum calcium & phosphate, liver function tests, DTH against common recall antigens, HIV Ab, thyroid function tests, Pregnancy test

B: **Toxicity assessment**: Clinical assessment, Full blood examination including differential white cell and , platelet count, Blood chemistry: serum urea, creatinine and electrolytes, serum calcium & phosphate, liver function tests.

C: in addition to B, also perform thyroid function tests.

D: **T-cell & immunology laboratory tests**: 150 ml in sterile tubes containing preservative free sodium heparin for tests of immune function.

E: **Dendritic cell phenotype:** 10 ml in a sterile tube containing preservative free sodium heparin.

F: **Detailed DC analysis including CFCs**:150 ml in sterile tubes containing preservative free sodium heparin.

G: 7.5 litre exchange to last approximately three hours. Final product volume of 50mL.

✣ Assessment of inflammatory responses: blood tests – see table in Appendix B.

Imaging: CXR, CT Chest, abdomen, and pelvis. Brain for initial staging only - thereafter, if clinically indicated.

* Including ophthalmic examination.

++ If present, photography of lesions and documentation of vitiligo should be performed at the commencement of each cycle.

7.0 PRE-TREATMENT AND TREATMENT EVALUATION

7.1 Pre-treatment tests

a Complete physical examination noting the size and location of any lesions.
b Hepatic, renal and thyroid blood chemistry panel.
c FBE differential, PT/PTT, platelet count.
d HIV Ab.
e Leukapheresis will be used to obtain peripheral blood lymphocytes in all patients on two occasions. It will consist of a 7.5 litre exchange to last approximately three hours.
f Cutaneous testing for recall antigens
g Tumour typing for antigen expression
h Baseline skin biopsy for DC populations

7.2 Schedule of Tests

Blood tests will be drawn according to the above schedule. To perform standard tests of important organ function and to isolate circulating haematopoietic progenitors, DC precursors and blood DC subsets mobilized or expanded by FL treatment for tests of immune responses.

To perform detailed tests of immune responses, Leukapheresis will be performed prior to the commencement of treatment and at the completion of vaccination/FL treatment. For patients completing three cycles of FL and vaccination, Leukapheresis will be performed on day 85. For patients who discontinue before the completion of three cycles, Leukapheresis will be performed at the time of discontinuation if the patient's clinical condition permits and at the discretion of the investigator.

A total of up to 1144 ml will be collected over the 3 month period. This comprises 50 ml for each Leukapheresis harvest and blood tests as outlined in the schedule of tests. The average volume removed will therefore be 130mL/week.

Results of all standard blood tests will be fully recorded on case report forms. Specialized assays of immunological function and DC phenotype will only be recorded in summary form.

Tests of immunological function will be performed in the laboratories of the Ludwig Institute for Cancer Research. A description of the methods which will be used appears in appendix B.

Patients will have Full Blood Examination, clinical evaluation, and routine blood tests each two weeks. Study agents can be given on an outpatient basis unless inpatient admission is indicated for underlying disease management.

Patients who develop inflammation, rashes, granulomas or ulceration at the site of vaccination may undergo biopsies or excision of these lesions. A separate consent form will be obtained if such biopsies are required.

Lesions may also be photographed at the discretion of the investigator

7.3 Tumour Responses

7.3.1 Complete response (CR): Disappearance of all signs, symptoms, biochemical and imaging evidence of tumour for a period of at least 30 days.

7.3.2 Partial response (PR): Decrease in size of all measurable or evaluable tumours by at least 50 % of the sum of the products of the greatest and perpendicular diameters, for at least 30 days, without appearance of new lesions or progression of any lesion.

7.3.3 Minor response (MR): Decrease in size of all measurable or evaluable tumours by at least 25 % of the sum of the products of the greatest and perpendicular diameters, for at least 30 days, without appearance of new lesions or progression of any lesion.

7.3.4 Stable disease (SD): No change in size of all measurable or evaluable lesions (less than 25 % of the sum of the products of the greatest and perpendicular parameters), for at least 30 days, without appearance of new lesions or progression of any lesion.

7.3.5 Progressing disease (PD): Appearance of new tumours, or increase in tumour size by at least 25% of the sum of the product of the greatest and perpendicular diameter.

7.4 Immunological Response

7.4.1 Humoral Immunity

Antibody against each antigen will be measured by ELISA (see Reference for methodology (Stockert 1998)).

7.4.2 Cellular Immunity

Antigen specific $CD8^+$ T-cell reactivity will be measured for each peptide antigen by:

- Tetramer analysis (Antigen specific; see reference for methodology: (Jager 2000)
- ELISPOT assay (Antigen-specific; see Reference (Jager 2000)

Antigen specific DTH will be measured by skin test (see Appendix B).

7.4.3 Dendritic Cell studies

Dendritic cells will be evaluated using methods to characterize phenotype and function (see Appendix B)

7.5 Other Evaluation Parameters

Antigen-presenting cell studies (see Schedule of Tests).

1 Skin:
Four mm punch biopsies of intradermal peptide injection sites and distant normal skin will be performed under local anaesthetic. Immunohistochemistry will be performed to define infiltrating mononuclear cells and Langerhans cells (CD3, CD4, CD8, HLA-DR, CD1a, and S100).

For patients in arm 3, skin will be biopsied from sites which have been treated with imiquimod but are not peptide vaccination sites. This will enable assessment of the effect of imiquimod on LC numbers.

2 Blood:

Detection of circulating peripheral blood monocytes and circulating DC by multi parameter flow cytometry (FACS). DC will be identified and their maturation status assessed using 4 color analysis. DC can be identified by correlating mature lineage marker negative cells (Lin-) (CD3, CD14, CD19 and CD56) with expression of CD33, HLA-DR, CD1b/c, CD86 or staining with CMRF44 (Hart, 1995). Alternatively, cells expressing CD11c but lacking CD14 are referred to as DC. CD45 will also be used to monitor haemopoietically-derived cells. Furthermore, expansion of DC lacking CD11c, previously referred to as plasmacytoid T cells (Grouard, 1997, Olweus, 1997) can be assessed by examining Lin- PBMC that are IL-3R+, HLA-DR+, CD4+, CD45RA+ but CD11c- and CD1b/c-.

In addition to the assays to assess T cell responses, and if sufficient cells are available, FL-generated DC will be cultured in the presence of various cytokines which may include, IL-1, IL-3, IL-4, IL-7, GM-CSF, TNF-alpha, CD40L, IFN-alpha, TGF-beta or LPS in order to study their ability to differentiate into mature activated DC in vitro. In vitro functional studies of these DC will also be performed using standard methods for evaluating endocytosis and phagocytosis.

8.0 STUDY COMPLETION, PATIENT EVALUABILITY, REMOVAL FROM STUDY

8.1 Study Completion

A patient will be considered to have completed the study following the week 15 follow-up clinical visit, at the time of death, or if lost to follow-up.

Patients will continue to be followed according to the schedule: at 6 and 12 months. This is to monitor for evidence of autoimmunity or disease relapse.

8.2 Patient Evaluability

Patients will be considered evaluable for immunological response and for tumour response endpoints after receiving 1 cycle of FL with or without 2 doses of peptide vaccination. Patients will be considered evaluable for toxicity after receiving one dose of FL.

8.3 Follow up

Follow up evaluation for disease progression and autoimmune adverse events are shown in the above table (schedule of tests – follow on phase).

At the discretion of the investigator, further vaccination with peptides alone may be offered to those patients who received the combination of FL plus peptide. The procedures and schedule of tests will follow those outlined above for weeks 5-8.

8.4 Criteria for Patient Withdrawal (Removal) from Study

In accordance with the Declaration of Helsinki, ICH Good Clinical Practice Guidelines and the US FDA Regulations, a patient has the right to withdraw from the study at any time for any reason without prejudice to his/her future medical care by the physician or at the institution. The investigator and Sponsor also have the right to withdraw patients from the study (see

EXHIBIT C

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIVISION OF ONCOLOGY

Protocol 102

1. A Phase I Study of a HER-2/neu Peptide-Based Vaccine with Flt3 Ligand or GM-CSF as an Adjuvant in Patients with Advanced Stage Prostate Cancer

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2. Introduction

Prostate cancer is the most common tumor among men, and the second leading cause of cancer-related death in men [1]. Despite advances in screening and early detection, however, there is projected to be a 37% increase in annual mortality over the next several years. Over 39,000 U.S. men are estimated to die as a result of prostate cancer this year [2]. Treatment with surgery and radiation remains effective for limited-stage disease, however approximately one third of patients will have progressive disease. There is currently no accepted adjuvant treatment for patients undergoing prostatectomy or ablative radiation treatment that has been shown to prevent or reduce the progression to metastatic disease. Prostate cancer, once it becomes metastatic, is not curable and is generally initially treated with androgen ablation therapy with an average three-year progression-free survival before the disease becomes refractory to hormonal manipulations. Once prostate cancer is hormone-refractory, treatment is generally palliative. In addition to new treatments for metastatic disease, new strategies are needed to prevent the progression to metastasis. A vaccine-based strategy offers hope for providing a safe, inexpensive treatment adjuvant to prevent the progression from limited-stage disease to metastatic disease, and a therapy targeting HER-2/neu (HER2) is particularly suited to preventing the progression to metastasis.

Attempts have been made over the last fifty years to develop vaccine strategies to aid in the treatment of various human malignancies, including prostate cancer. In the past, these approaches utilized inactivated autologous tumor cells, based on the model of vaccinating individuals against infectious diseases using inactivated pathogens. In general, these approaches with autologous tumors met with limited success. Recent advances in basic immunology, such as improved understanding of antigen presentation, how T cells recognize antigens, and the role of various cytokines in the generation of an immune response, suggest novel means of generating immunity against autologous tissues. In addition, recent advances in molecular biology have led to the identification of tumor-associated antigens and the identification of novel cytokines acting in the immune response. Such advances have allowed the identification of vaccine strategies that may be more effective in the treatment of human cancers. Specifically, the ability to target a tumor antigen should permit one to generate an effective anti-tumor T cell population, rather than non-specifically

stimulating the immune system. Several lines of evidence have pointed to the importance of cytotoxic T lymphocytes (CTL) in tumor protection [3,4]. In general, however, it has been difficult to generate tumor antigen-specific CTL using vaccines. Studies in a rat model of autoimmune prostatitis have shown the importance of a CD8+ CTL response to the development of inflammatory prostatitis with tissue destruction [5]. Recently, several cytokines have been identified which have the potential to create an immune environment conducive to the generation of CD8+ CTL. This study outlines a phase I trial of a peptide-based cancer vaccine directed against the HER2 oncogenic protein, using flt3 ligand (FL) or granulocyte-macrophage colony stimulating factor (GM-CSF) as adjuvants, in an attempt to specifically generate a HER2-specific CTL response in patients with prostate cancer.

3. Background

A. HER2 is a tumor antigen for prostate cancer:

The HER2 protooncogene encodes a 185 kD transmembrane protein with homology to epidermal growth factor receptor [6]. The HER2 protein consists of a cysteine-rich extracellular domain which presumably functions as a growth factor receptor, a short transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity. Whereas many protooncogenes are activated by mutations, and indeed the rat homologue *neu* first identified in rat neuroblastomas was shown to have a point mutation in the transmembrane domain, human HER2 protein does not appear to be transforming by mutation but rather by gene or protein overexpression [7]. HER2 gene amplification and/or protein overexpression occurs in many adenocarcinomas including breast, ovary, colon, and lung [8]. The amount of HER2 overexpression in prostate adenocarcinoma has been the subject, however, of some debate, with different groups reporting anywhere from 16 to 100% overexpression, but all with lower tissue expression than that seen in primary breast and ovarian cancers [9,10,11]. At the University of Washington, analysis of over 30 primary prostate adenocarcinomas has revealed no overexpression by immunohistochemistry, using the same criteria accepted for breast and ovarian cancer. On the contrary, about half of prostate metastases analyzed here at the University of Washington have shown HER2 overexpression, to the same level detected in breast and ovarian primary tumors [True, personal communication]. When the primary tumors have been reevaluated in some of these same patients, there was no identifiable overexpression of HER2, suggesting that this is a phenomenon that occurs during the metastatic progression of the disease. This is consistent with the recently reported observation that HER2 overexpression in prostate cancer cells can overcome the androgen-dependent growth inhibition and lead to androgen-independent growth [12]. While it is clear that HER2 overexpression in ovarian and breast cancer portends a poorer prognosis with shorter survival [13,14], the situation is less clear with prostate cancer. Initial reports suggested a correlation of HER2 expression with prostate cancer tumor grade, a known prognostic indicator [9,15,16]. Other reports found no correlation with tumor grade, or perhaps even an inverse relation with Gleason scoring [11]. Given the high degree of HER2 expression in breast ductal carcinoma *in situ* and the variable expression in primary prostate adenocarcinomas, it has been suggested that HER2 is involved in the malignant transformation process [11]. The recent findings that HER2 overexpression can lead to androgen-independent growth also suggest that HER2 is involved in the progression to metastatic disease, but is variably expressed in primary tumors [12]. A similar study in an animal model demonstrated that a rat prostate cancer cell line transfected with a gene encoding rat *neu* had substantially enhanced tumorigenicity and metastatic potential, again suggesting that the human HER2 homologue is indeed involved in the acquisition of tumorigenic and metastatic potential of prostate cells [17]. As a putative growth factor receptor overexpressed on the surface of malignant cells and as a potential factor in the malignant transformation of prostate tissue, HER2 may be an excellent target for immune-based treatment strategies with the goal of preventing the progression to more advanced disease.

B. CTL and antibody responses specific for HER2 can be detected in patients with cancers overexpressing HER2:

Approximately 25% of patients with breast cancer have tumors that express HER2 [18]. Of these patients, approximately 20% have a detectable humoral immune response to HER2 [19]. Similar results have been found in patients with colon cancer (in press), and we have identified HER2-specific IgG antibodies in 17% of patients with prostate cancer, irrespective of the expression HER2 by the primary tumor [McNeel, manuscript in preparation]. Other groups have identified CTL that recognize HER2 in patients with breast or ovarian cancers overexpressing HER2 [20,21]. The observation that immunity in patients co-exists with antigen-positive cancer cells indicates that HER2 is immunogenic and immunization occurs by exposure to the overexpressed protein on cancer cells. In these studies, CTL recognizing HER2 could only be detected after multiple *in vitro* enrichment steps. This suggests that the level of existent immunity in patients may be inadequate to eliminate the cancer cells. Previous work in our lab has focused on developing a vaccine strategy to initiate or boost immunity to the HER2 protein in patients with HER2-expressing breast, ovarian or lung cancer. The purpose of the current protocol is to develop a vaccine capable of eliciting and/or substantially augmenting CTL to the HER2 protein in patients with advanced stage prostate cancer.

C. Peptide epitopes derived from the HER2 protein sequence can be immunogenic:

The current protocol will examine the use of a peptide derived from the HER2 protein to induce and/or boost immunity, and specifically CTL, to HER2. The presumption is that immunization with peptides is more effective than immunization with whole protein [22]. In addition to the theoretical concern of being able to overcome immune system tolerance to self oncogenic proteins, peptides have additional advantages including: (1) peptides are a chemically defined product, therefore, no infectious agents or nucleic acids are present in the final products or during synthesis, (2) their use will allow the elimination of epitopes that may include suppresser determinants, (3) one has the potential to tailor the immune response to only those epitopes which can elicit certain immune responses, such as the generation of CTL.

In general, it is known that peptides of 9-10 amino acids in length preferentially bind to MHC class I molecules for the elicitation of CTL. Several HLA-A2 CTL epitopes have been defined for HER2 [20,21,23]. The peptide p369.9 (amino acids 369-377: KIFGSLAFL, also known as E75 peptide), in particular, is a well-characterized HER2 epitope known to be processed and presented by HER2-positive tumor cells and recognized by HLA-A2-restricted CTL [20,24]. Previous work has shown that CTL lines recognizing the p369.9 peptide are able to lyse tumors expressing HER2 [24].

D. Results from a phase I study using a HER2 peptide-based vaccine to augment immunity in patients with HER2-expressing breast, ovarian, or lung cancer.

Since September of 1996 we have been conducting a phase I study of three HER2 peptide based vaccine formulations with GM-CSF as the adjuvant for patients with advanced stage HER2-expressing breast, ovarian or lung cancer [25]. As part of this study, 18 patients (17 with stage III or IV breast cancer and 1 patient with ovarian cancer) have received vaccinations with a mixture of three 15-mer HER2 HLA-A2-restricted CTL peptide epitopes. One of the 15-mer peptides used in this vaccine encompassed the p369.9 peptide sequence as well as a putative CD4 helper epitope. The three peptides were mixed together and administered with 150 µg GM-CSF intradermally monthly for 6 months. Other than mild transient myalgias and urticaria, as well as transient erythema and induration at the injection sites, there has been no significant toxicity seen in this group of patients. Delayed-type hypersensitivity (DTH) testing to each of the individual immunizing peptides was performed after the administration of 6 vaccinations. Eleven patients who completed the entire vaccination series have been evaluated for DTH responses to p369. 9 of these 11 patients

demonstrated a positive DTH response to p369 (5 had > 10 mm of response, 4 had 5-9 mm, and 2 had <5 mm responses. Skin biopsies of the DTH site showed infiltration of CD3+ T cells comprised of both CD4+ and CD8+ subpopulations. After the course of immunizations we have also been able to demonstrate HER2 peptide- and protein-specific CTL. Figure 1 is an example of such data obtained for a single patient throughout the course of the study. Patient LAD0107 had no evidence of CTL activity in peripheral blood mononuclear cells (PBMC) to target cells pulsed with the HER2 HLA-A2 binding peptides in her immunizing mix or to HLA-matched tumors overexpressing the HER2 protein prior to immunization. 30 days after the second immunization, however, it was possible to detect HER2-specific CTL activity. PBMC from this patient was taken through a single *in vitro* stimulation with 1.0 μM each of the 9-mer HLA-A2-binding HER2-derived peptides encompassed in the CTL vaccine. In a 4-hour chromium release assay (CRA) the patient demonstrates lytic activity directed against target cells pulsed with two peptides in the vaccine, p369.9 and p688.9. In addition, CTL recognize SKOV3 A2, a HER2-overexpressing tumor cell line that is transfected with HLA-A2. The T cells do not lyse the parental line, SKOV3, which does not express HLA-A2, demonstrating that this cell-mediated lysis is HLA-restricted. In summary, our previous experience immunizing patients with HLA-A2-restricted HER2-derived peptides has resulted in enhanced cellular immunity to HER2 as demonstrated by positive DTH responses, CD4+ T cell proliferative responses, and with generation of CTL capable of lysing HER2-expressing tumor cells *in vitro*. Furthermore, this treatment has demonstrated no significant toxicity.

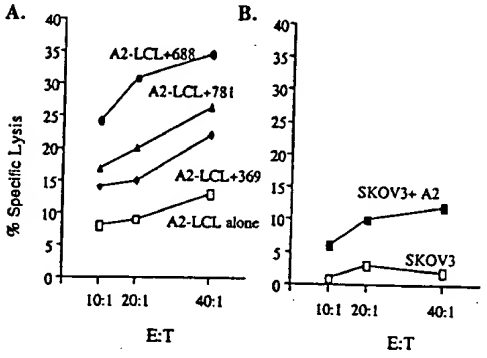

Figure 1. Patients immunized with HER2 peptides develop HER2 peptide- and protein-specific CTL responses. Data is shown from a patient, LAD0107, immunized with the CTL peptide vaccine. Panel A: PBMC was taken from the patient 30 days after the second immunization. $20x10^6$ cells were bulk cultured with 1.0 μM each of the HLA-A2 binding 9-mers in the immunizing vaccine. Cells were cultured for 10 days and IL-2 5 U/ml was added throughout the cycle on days 3, 6 and 9. After 1 IVS, the lines were tested for lytic activity in a 4-hour CRA against homozygous allogeneic HLA-A2 LCL alone and HLA-A2 LCL coated with HER2 HLA-A2 CTL binding peptides. Panel B: T cell lines were also evaluated for their ability to lyse SKOV3, a tumor cell line which overexpresses the HER2 protein, and SKOV3 transfected with HLA- A2.

E. Cytokines can be used as adjuvants in humans:

GM-CSF

GM-CSF has been studied extensively by ourselves and others in animal and human models as a vaccine adjuvant. GM-CSF is a known growth and differentiation factor for human dendritic cells [26,27]. Dendritic cells are specialized antigen-presenting cells that are believed to be responsible for stimulating naïve T cell responses. They have also been shown in experimental models to augment secondary immune responses better than other antigen-presenting cells. The dermis is a site for skin dendritic cells (Langerhans cells) that are important in initiating early immune responses by migrating to draining lymph nodes after being exposed to antigen and presenting the antigen to T cells. To test the effect of GM-CSF as an adjuvant, we immunized rats with a foreign protein, tetanus toxoid, in a suboptimal fashion (low dose and single injection) [28]. GM-CSF or PBS was mixed with the antigen and both were injected intradermally on days 1 and 2. The immunizations were administered at the same site each day for potential stimulation of dermal dendritic cells. For the subsequent three days animals received either GM-CSF or PBS intradermally. After 2 weeks, the rats were evaluated for their response to tetanus toxoid. All animals immunized with GM-CSF as adjuvant developed significant IgG antibody responses against tetanus. None of the animals

immunized with tetanus in saline developed an antibody response. A similar strategy was used immunizing animals with peptides derived from the rat *neu* protein that had been previously shown to generate an immune response in rats when CFA was used as an adjuvant. Rats immunized with the peptides and GM-CSF developed evidence of a DTH response to the peptides after 1 immunization whereas animals that received peptides in saline did not [28]. Similar studies in the laboratory were able to elicit similar responses by using a single dose of soluble GM-CSF admixed with peptides and injected intradermally.

Similarly have been found in human studies using GM-CSF as a vaccine adjuvant. In a phase I study of GM-CSF as an adjuvant for a hepatitis B vaccine, doses of 20-80 µg were administered just prior to and at the same site as the vaccination. Of 81 subjects treated with GM-CSF, 15 developed anti-HBs after only 1 injection (10 had protective titers) while only 1 of 27 patients vaccinated without GM-CSF produced a weak and transient antibody response [29]. In a pilot study of 15 hemodialysis patients who failed to respond to a hepatitis B vaccine, subjects were given a single dose of GM-CSF 24 hours before revaccination. 1 of 6 patients who received 0.5 µg/kg, 4 of 5 who received 5 µg/kg and 2 of 4 who received 10 µg/kg GM-CSF responded with protective levels of anti-HBs [30].

FL

FL has been shown to be a potent stimulator of early hematopoietic cells, and in particular for the generation of progenitor dendritic cells [31]. Previous studies have demonstrated that FL administered systemically to mice can markedly increase circulating progenitor dendritic cells [32,33] that retain antigen-presenting function and the capacity to stimulate the proliferation of antigen-specific T cells [34]. Administration of FL alone, in fact, could confer resistance to a murine C3L5 breast cancer challenge [32]. Similarly, systemic administration to mice after challenge with B16 or EL-4 tumors resulted in significant decreases in tumor growth rates, with infiltration of dendritic cells noted within tumor tissue sections [35]. Recombinant human FL has been safely given to normal human volunteers and has been used to expand human dendritic cells *ex vivo* from peripheral blood stem cells [36]. The ability of FL to stimulate the production of dendritic cells suggests it may be useful as a vaccine "adjuvant" by increasing the number of circulating and tissue-resident antigen presenting cells. The increased number of dendritic cells may thereby increase the presentation of a peptide vaccine antigen and promote a more vigorous T cell response. The current study will evaluate the use of FL in this novel role as a potential vaccine adjuvant, and compare the administration of a peptide antigen given intradermally at two different time points during the mobilization of dendritic cell precursors using FL. Results from preliminary animal studies suggest that FL is useful as a vaccine adjuvant with the ability to steer the immune response towards a Th1-type immune response [Smorlesi, unpublished observations]. The ability to function as an adjuvant, however, likely depends upon the maturity of dendritic cells for proper antigen processing and presentation, and therefore the proper time of immunization, not known at present, will be investigated by immunizing at either day 7 or day 14 of the FL cycle.

4. Objectives

A. Primary Objective:

To determine the safety and feasibility of serial intradermal vaccinations of a HER2-derived HLA-A2-restricted peptide, p369.9, with FL or GM-CSF as an adjuvant in patients with advanced stage prostate cancer.

B. Secondary Objectives:

1. To determine whether HER2 peptide-specific and tumor-specific CTL can be generated in patients with advanced stage prostate cancer.

2. To evaluate the efficacy of GM-CSF or FL as adjuvants for the generation of HER2-specific CTL in patients with advanced stage prostate cancer.

5. Vaccine Preparation

A. HER2 derived peptide:

p369-377 KIFGSLAFL

B. Peptide synthesis and characterization:

The peptide is provided by Corixa Corporation, Seattle, WA. Peptide was manufactured by Multiple Peptide Systems, San Diego, CA. using standard solid phase synthesis techniques employing Boc chemistry. Amino acids and resins for synthesis were purchased from Bachem California and the amino acids were analyzed by TLC and polarimetry prior to use. The peptide was cleaved from the solid support using anhydrous hydrogen fluoride and conventional techniques. Peptide was purified by HPLC using a Waters Delta-prep system with a gradient of acetonitrile (containing 0.1% trifluoroacetic acid) in water (also containing 0.1% trifluoroacetic acid). It was then lyophilized to dryness before being passed over an anion exchange column to exchange the trifluoroacetate for acetate. GLP procedures were followed for the manufacture of the peptide and FDA approval has been obtained for use of this peptide prior to initiating its' use in humans.

The peptide was characterized by mass spectrometry to confirm its identity, by HPLC for purity using two different elution buffers, and by amino acid analysis for identity and peptide content. The water content was determined by the Karl Fischer procedure and the data obtained used to verify the mass balance.

Prior to formulation, the solubility of the peptide in 10 mM sodium acetate buffer (pH 4.0) was determined, and then diluted to a concentration of 833 µg/ml. This will allow the total vaccine dose to be 0.5 mg in 0.6 ml using 2 injections of 0.3 ml. The stability of the peptide in the acetate buffer was monitored by HPLC and mass spectrometry. The peptide was vialed under sterile conditions and microbial and sterility testing were conducted to ensure safety. The dissolved peptide was then stored at –20°C prior to use.

C. GM-CSF:

GM-CSF (Sargramostim), supplied by Immunex Corporation, Seattle, WA, will be used as a vaccine adjuvant. GM-CSF is a growth factor that supports the survival, clonal expansion and differentiation of hematopoietic progenitor cells including dendritic antigen presenting cells. In preliminary animal experiments GM-CSF served as an effective adjuvant for the induction of antibody and CD4+ T cell responses [28]. The use of GM-CSF is associated with little toxicity [26,27,29]. GM-CSF is a sterile, white, preservative-free, lyophilized powder supplied in 500 µg-dose vials. Vials will be reconstituted with the peptide solution (already in a 1 ml volume of 10 mM sodium acetate buffer). Reconstituted GM-CSF (0.2 ml) with peptide solution (0.6 ml) will be injected i.d. for a total dose of 0.8 ml for each immunization. The volume of vaccine will not exceed 0.4 ml per

each of 2 injection sites. Thus, for the current study, GM-CSF will be used at a total injection dose of 150 µg mixed prior to injection with peptide and injected intradermally at the time of immunization.

Recombinant human GM-CSF (rhGM-CSF), when administered intravenously or subcutaneously is generally well tolerated at doses ranging from 50 to 500 µg/m^2/day. Severe toxic manifestations are extremely rare in patients treated with rhGM-CSF.

Diarrhea, asthenia, rash and malaise were the only events observed in more than 5% of the rhGM-CSF group compared with the placebo group in phase III controlled studies of patients undergoing autologous bone marrow transplantation. In uncontrolled phase I/II studies for various indications, the adverse effects reported most frequently were fever, chills, nausea, vomiting, asthenia, headache and pain in the bones, chest, abdomen, joints, or muscles. Most of these systemic events reported with rhGM-CSF administration were mild to moderate in severity and rapidly reversed by the administration of analgesics or antipyretics. Other events reported infrequently were dyspnea, edema, local injection site reactions, and rash. Thrombosis and cardiac arrhythmia have also been reported, and there have been infrequent reports of tissue sloughing, leukemic progression, congestive heart failure, hepatomegaly, and intercranial bleeding; and isolated reports of Guillain-Barré syndrome and increased histiocytes. Neutropenia, although usually a pre-existing condition in the patients receiving rhGM-CSF, has been reported in association with administration. There was an increased frequency of severe thrombocytopenia in patients receiving concurrent chemotherapy and radiotherapy with GM-CSF. Eosinophilia and other blood abnormalities may occur.

There have been rare reports of 1) sequestration of granulocytes in the lungs with respiratory symptoms; 2) a syndrome characterized by respiratory distress, hypoxia, flushing, orthostatic hypotension, and partial loss of consciousness; 3) peripheral edema, pericardial or pleural effusions, and capillary leak of fluid; and 4) serious allergic or anaphylactic reactions.

Administration of rhGM-CSF may aggravate fluid retention in patients with pre-existent edema, capillary leak syndrome, or pleural or pericardial effusions. In some patients with pre-existing renal or hepatic dysfunction, elevation of the serum creatinine or bilirubin and hepatic enzymes has occurred during the administration of rhGM-CSF. Dose reduction or interruption of rhGM-CSF administration has resulted in a decrease to pretreatment values. Occasional transient and reversible supraventricular arrhythmia has been reported in uncontrolled studies, particularly in patients with a previous history of cardiac arrhythmia.

Stimulation of marrow precursors with rhGM-CSF may result in a rapid rise in white blood cell count. Dosing should be stopped if the ANC exceeds 20,000/cm^3. rhGM-CSF may stimulate the growth of myeloid malignancies; therefore, caution must be exercised in its use in these malignancies or myelodysplastic syndromes.

Because GM-CSF is only administered monthly in one arm of patients on the current protocol, most of these side effects are not anticipated. In our previous vaccine study using rhGM-CSF as an adjuvant in an identical fashion, we did see local skin reactions consisting of erythema and induration in 17% of the patients. This resolved without need for treatment over a 2-3 week period. We have also observed transient leukopenia in some patients without clinical sequelae, and which resolved without treatment. We and others have also noted the generation of a transient immune response to rhGM-CSF following its use in similar fashion, however no clinical sequelae were noted, and the significance of this observation is not clear at present.

C. FL:

FL, supplied by Immunex Corporation, Seattle, WA, will be used as a vaccine adjuvant. Previous studies in normal human volunteers have shown that doses up 100 µg/kg/d s.c. for 14 days were well

tolerated [37]. Previous studies looking at the generation of dendritic cells showed that increased dendritic cells could be detected in the peripheral blood beginning shortly after daily administration, with peak levels of circulating dendritic cells seen on about day 9 of daily administration. Levels of peripheral dendritic cells remained elevated until approximately day 21 after cessation of treatment on day 14. For the current study, patients receiving FL will receive a daily dose of 20 µg/kg (up to a maximum of 1500 µg/day) administered subcutaneously daily for 14 days, followed by a 14-day rest period. FL is supplied as a sterile solution in 1500 µg single-use vials. Patients receiving FL will receive their first daily dose under observation, for training and instruction in self-administration.

Commonly expected side effects associated with FL administration have included erythema and induration at the site of injection. Bruising, pain, blistering and pruritis at the injection sites have also been reported. Increases in peripheral white blood cell counts, especially monocytes, are expected, as are increases in serum enzymes (such as LDH) that are associated with leukocytosis. Increases in liver function tests (AST and ALT) are less frequently seen. Enlarged, painful lymph nodes have also been reported. All of these side effects are expected and were predicted in animal models. They were reversible with discontinuation of the drug.

In addition, with FL there have been infrequent reports of back and muscle pain, malaise, nausea, diarrhea, paresthesias, pharyngitis, headache, rhinitis, herpes simplex stomatitis, dyspepsia, and hematuria. These events may or may not have been related to the drug. Side effect reports following the administration of FL in combination with G-CSF or GM-CSF (which will not be the case in this particular study) have included constipation, albuminuria, emesis, conjunctivitis, dizziness, hypotension, fever, sweats, and rare cases of hypercoagulability. These events may or may not have been related to FL. Although there is no evidence in animal models that healthy animals develop autoimmune disease following FL administration, it is unknown whether FL may exacerbate autoimmune disorders. Therefore, subjects with a personal history of autoimmune disease will not be enrolled to receive FL.

Furthermore, some drugs used to treat cancer have an inherent risk of causing secondary cancers and/or leukemias. Although there is no evidence to date that FL has an inherent risk of causing such problems, because it is a growth factor for hematopoietic progenitor cells this remains a theoretical risk. Likewise, because of the investigational nature of this drug, there may be other unanticipated side effects that have not yet been reported.

6. Patient Selection

A. Inclusion Criteria

1. Patients with stage C or D adenocarcinoma of the prostate.
2. Patients must be HLA-A2 positive.
3. Androgen deprivation therapy will be allowed as a concurrent treatment modality.
4. Patients should have an expected survival of at least 12 months and a Karnofsky performance score > 80%.

B. Exclusion Criteria

1. Patients cannot be on immunosuppressive therapy, such as chemotherapy, chronic treatment dose corticosteroids, or radiation therapy for bony metastases, within four weeks of the first dose of cytokine or cytokine + peptide.
2. WBC < 3000/mm^3, platelet count < 100,000/mm^3, serum creatinine > 1.5 mg/dl or a calculated creatinine clearance < 60 cc/min, serum bilirubin > 2.0 mg/dl.
3. History of autoimmune disease.

4. History of hematologic malignancy.
5. Concurrent enrollment on other phase I, II, or III investigational studies.

7. Experimental Design

There will be 4 study arms with 5 patients enrolled in each arm. A total of 20 patients with stage C or D prostate cancer will be enrolled.

ARM 1: FL alone. FL at 20 µg/kg/day s.c. for 14 days followed by a 14-day rest period. FL alone will be administered as described for a total of four cycles.

ARM 2: FL + p369.9 (500 µg) given as a single i.d. injection on day 7 of the 14-day FL administration. There will then be a 14-day rest period. This mode of vaccination will be repeated for a total of four 28-day cycles.

ARM 3: FL + p369.9 (500 µg) given as a single i.d. injection on day 14 of the 14-day FL administration. There will then be a 14-day rest period. This mode of vaccination will be repeated for a total of four 28-day cycles.

ARM 4: rhGM-CSF (150 µg) + p369.9 (500 µg) mixed together and given as an i.d. immunization on day 1 of a 28-day vaccine cycle. This cycle will repeated for a total of four 28-day cycles.

Patients will be assigned to arms 1-4 in a random fashion.

All patients will undergo leukapheresis at beginning and after 4 vaccinations to insure adequate PBMC for the laboratory component of the study. Chemical and clinical parameters will be evaluated monthly to determine systemic toxicity.

8. Criteria for Premature Study Termination

Patients will be enrolled on the study as outlined in section 7. If at any time during the study, there is sufficient evidence suggesting that the Grade 3 or Grade 4 toxicity rate is excessive, then the study will be terminated. ARM 1-4 will be evaluated as separate populations. Toxicity will be measured by the modified NCI Common Toxicity Criteria set in Appendix D. An exception will be made for grade 3 skin toxicity, as generalized rash with some mild symptoms are expected in patients treated with FL.

An excessive Grade 3 toxicity rate will be taken to be 20% and an excessive grade 4 toxicity rate will be taken to be 10%. Evidence that the toxicity rate is excessive will be considered sufficient if the lower limit of the 90% one-sided confidence interval for the estimate of the true toxicity rate exceeds the appropriate limit (20% for Grade 3, 10% for Grade 4). Operationally, this will occur if any of the following occur:

Grade 3 toxicity: Present in 2 of 2 patients, 3 of 5 patients.
Grade 4 toxicity: Present in 1 of 2 patients, 2 of 5 patients.

In addition, patients requiring corticosteroid therapy, radiation therapy, or chemotherapy for the treatment of their underlying prostate cancer while on study will be removed from the study.

9. Management of Toxicities and Complications

If a patient develops grade 3 toxicity or greater related to the immunizations, no further vaccinations will be given to that patient. If the toxicity observed is felt to be related to the immune response generated, a regimen of corticosteroids will be administered. The following dose schedule will be used:

Day 1: Intravenous Solu-Medrol at 1 mg/kg IV q12 hr
Day 2: Intravenous Solu-Medrol at 1 mg/kg IV x 1
Day 3-4: Prednisone at 30 mg p.o. bid
Day 5-6: Prednisone at 15 mg p.o. bid
Day 7-8: Prednisone at 10 mg p.o. bid
Day 9-10: Prednisone at 10 mg p.o. qd
Day 11-12: Prednisone at 5 mg p.o. qd

Although unexpected, any patient requiring IV corticosteroids will be monitored on the CRC unit of the University of Washington Medical Center until resolution of symptoms.

10. Plan of Treatment

The following section describes the schedule for prescreening, vaccine inoculations, clinical and laboratory evaluations. When a chemistry panel is indicated the following tests are performed: sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, SGOT, SGPT, alkaline phosphatase, and LDH. This plan is summarized in Appendix B.

A. Prescreen:

HLA tissue typing to confirm HLA-A2 positivity.

B. Initial Evaluation:

1. Sign consent form.
2. Medical history and complete physical examination. Clinical evaluation, including vital signs symptoms assessment, and Karnofsky score (Appendix C).
3. Leukapheresis procedure to obtain PBMC for the evaluation of preexisting cellular immunity to the HER2 protein, peptide and adjuvant by T cell proliferative assays and by CTL CRA. PBMC will also be used to assess precursor frequencies of HER2-specific CTL by ELISPOT.
4. Obtain 10 ml. of peripheral blood for the evaluation of a preexisting antibody response to HER2 protein, peptide and the adjuvant.
5. Evaluation of blood cell counts (CBC), chemistry panel, serum prostate specific antigen (PSA), serum prostatic acid phosphatase (PAP), anti-nuclear antibody screen (ANA), thyroid stimulating hormone (TSH), and urinalysis.
6. DTH skin testing using sterile water, adjuvant (100 µg either rhGM-CSF or FL), p369.9 peptide (100 µg), and a panel of standard DTH antigens as baseline assessment. These tests will be performed on the back of subjects, and skin biopsy of baseline positive responses will be performed.
7. Patients will be assigned in a random fashion to one of the four treatment arms, as documented above.

C. Monthly immunizations:

1. Monthly brief physical examination, with symptom assessment, toxicity assessment and Karnofsky score.
2. Patients will receive monthly immunizations as described in sections 5 and 7 above. Specifically, 0.5 mg (0.6 ml of the 0.833 mg/ml) p369.9 peptide vaccine solution will be administered intradermally for patients on arms 2-4. Peptide admixed with 0.2 ml of reconstituted rhGM-CSF (100 µg) will be given to patients on arm 4. Patients receiving FL will be seen on the first day of the monthly FL administration and on the day of peptide vaccine administration.
3. Following vaccinations, the subjects will be observed for 60 minutes. Temperatures and reactions will be assessed by the staff at the end of the post immunization period. Subjects will take and record their temperatures the same evening (and at additional times if they have symptoms of fever) and record any other symptoms they experience. DTH responses will be assessed at 24-48 hours. Skin changes will be reviewed and documented at each visit. Unanticipated skin rashes will be biopsied for tissue evaluation.
4. Monthly CBC, PSA, PAP and chemistry panel (to be obtained on the first day of each 28-day cycle).
5. Patients on all arms will receive 10-20 cc blood draws on the day of peptide vaccination, and those patients receiving FL will also have an additional 10-20 cc blood draw on day 1 of FL administration. These samples will be used for assessing peripheral blood dendritic cell mobilization.
6. On the first day of the third immunization cycle, 80 cc of blood will be drawn to prepare PBMC and sera for the evaluation of augmentation or generation of an immune response to HER2. Specifically, T cell proliferative assays will be used to evaluate a T cell response to HER2 protein or peptides, and ELISA will be used to detect an antibody response to HER2 protein and peptides.

D. Final Evaluation:

1. 48-hour DTH skin testing using sterile water, adjuvant (100 µg either rhGM-CSF or FL), p369.9 peptide (100 µg), with skin biopsy of DTH responses. These DTH tests and biopsies will be performed on the back of subjects.
2. Leukapheresis procedure to obtain adequate numbers of PBMC for the evaluation of elicitation or augmentation of cellular immunity to the HER2 protein, peptide and adjuvant by T cell proliferative assays and standard CTL CRA, and to assess precursor frequencies of HER2-specific CTL by ELISPOT.
3. Obtain 10 ml of peripheral blood for evaluation of antibody responses to HER2 or adjuvant.
4. Evaluation of CBC, chemistry panel, PSA, PAP, ANA, TSH and urinalysis.
5. Where feasible, obtain 80-ml blood samples for the preparation of PBMC and sera every 2-3 months for up to one year following the last vaccination to evaluate retention or augmentation of cellular and humoral immunity.

11. Immunologic Monitoring

See Appendix A

12. Reporting Adverse Events

A. Definition:

EXHIBIT D

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIVISION OF ONCOLOGY

Protocol 104

1. A Phase I Study of a HER-2/neu Peptide-Based Vaccine with Flt3 Ligand with or without rhGM-CSF as an Adjuvant in Patients with Advanced Stage HER2-Expressing Breast or Ovarian Cancer

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2. Introduction

Advances in molecular biology have revolutionized vaccine research. However, tumor antigens (peptides and proteins) are often weak immunogens [1]. Cytokines have been shown to be efficient vaccine adjuvants in that they can enhance the immunogenicity of vaccines and potentially direct the immune response to a cytotoxic T cell (CTL), T-helper (Th) 1 or 2 subset response or B cell response. To that end, rhGM-CSF has been studied extensively as an adjuvant for vaccines in that it is known to promote proliferation of macrophages, increase MHC class II expression and increase dendritic cell (DC) maturation and prolferation both peripherally and locally. We have previously shown in a rodent model that GM-CSF can be used as a vaccine adjuvant when mixed with a soluble antigen to induce both an antibody and T cell antigen specific immune response [2]. Currently, we have an ongoing phase I study using rhGM-CSF as adjuvant with a HER-2/neu (HER2) peptide-based vaccine for patients with tumors that overexpress HER2. Flt3 ligand is a cytokine that has been shown to produces large numbers of DC in lymph nodes, peripheral blood, bone marrow, spleen, liver and skin [3]. The purpose of the current protocol is to see if using FL as an adjuvant to manipulate the APC milieu will substanially augment HER2 specific T cell immunity.

Specifically, this study outlines a phase I trial of a peptide-based cancer vaccine directed against the HER2 oncogenic protein using Fl, with or without rhGM-CSF, as adjuvant(s) in an attempt to generate HER2 specific Th and CTL responses in patients with HER2 expressing breast or ovarian cancer.

3. Background

A. HER2 is a tumor antigen for breast and ovarian cancer

The HER2 protooncogene encodes a 185 kD transmembrane protein with homology to epidermal growth factor receptor [4]. The HER2 protein consists of a cysteine-rich extracellular domain (ECD) which presumably functions as a growth factor receptor, a short transmembrane domain, and a cytoplasmic or intra-cellular domain (ICD) with tyrosine kinase activity. Whereas many protooncogenes are activated

by mutations human HER2 protein does not appear to be transforming by mutation but rather by gene or protein overexpression [5].

B. Preliminary studies in neu-t mice demonstrate protection from tumor with an ICD peptide vaccine:

Neu transgenic mice are engineered to express non-transforming rat neu on an MMTV promoter [6]. These animals develop neu overexpressing breast cancer at about 100-200 days of life. The breast cancer which occurs in these mice is histologically similar to the cancer which occurs in humans. Hyperplastic lesions progress to infiltrating ductal carcinomas which commonly metastasize to local lymph nodes and soft tissue sites. Only 80% of mice, however, will develop these neu overexpressing breast cancers. In addition, the time frame of tumor development varies greatly from mouse to mouse. We developed a tumor transplant system with naturally occurring neu mediated tumors in the neu-t mice. Transplanting tumors from older mice into younger age matched littermates generates 100% tumor uptake within 2 weeks of implant. In addition, the neu protein remains overexpressed in the transplanted tumors and which have metastatic patterns similar to those seen in spontaneously occurring neu-t tumors.

Rat neu protein in neu-t mice is a self protein. Previous studies, in rats, have shown that tolerance to rat neu can be circumvented with a peptide based vaccine [7]. We immunized neu-t mice with a vaccine composed of peptides derived from the ICD of rat neu. This same peptide vaccine could generate a rat neu protein response in rats. Similarly, in neu-t mice, where rat neu is a self protein, immunization with the peptide based vaccine resulted in the generation of rat neu specific immunity.

Mice immunized twice with the ICD vaccine and then implanted with rat neu expressing breast cancers reject the tumors. In these animals, the vaccine was protective but only if mice had immunity at the time they were given the tumor challenge. If animals began the immunization schedule at the same time as the tumor was inoculated, e.g. immunizing with a substantial tumor burden, there was no effect of peptide immunization in slowing tumor growth as compared to adjuvant alone.

C. rhGM-CSF as adjuvant:

Functionally, GM-CSF is thought to stimulate dendritic cell maturation and the up-regulation of epidermal APCs (Langerhans cells) which funtionally migrate to draining lymph nodes after being exposed to antigen and present antigen to T cells.

GM-CSF has been studied extensively by ourselves and others in animal and human models as a vaccine adjuvant. We have previously shown that rhGM-CSF, when mixed with soluble antigen, can act as adjuvant in the induction of both antigen specific antibodies and antigen specific Tc responses in the rodent model [2].

In a phase I study of rhGM-CSF as an adjuvant for a hepatitis B vaccine, doses of 20-80 μg were administered just prior to and at the same site as the vaccination. Of 81 subjects treated with rhGM-CSF, 15 developed anti-HBs after only 1 injection (10 had protective titers) while only 1 of 27 patients vaccinated without rhGM-CSF produced a weak and transient antibody response [8]. In a pilot study of 15 hemodialysis patients who failed to respond to a hepatitis B vaccine, subjects were given a single dose of rhGM-CSF 24 hours before revaccination. 1 of 6 patients who received 0.5 μg/kg, 4 of 5 who received 5 μg/kg and 2 of 4 who received 10 μg/kg rhGM-CSF responded with protective levels of anti-HBs [9].

D. Phase I Study of a HER2 peptide based vaccine using GM-CSF as adjuvant in patients with tumors overexpressing HER2:

Since September of 1996 we have been conducting a phase I study of three HER2 peptide based vaccine formulations each admixed with 250 μg rhGM-CSF as the adjuvant. To date 70 patients with stage III or IV breast cancer (n= 57), ovarian (n= 11) or non small cell lung cancer (n=2) have enrolled on trial. Patients are vaccinated monthly for six months with one of the 3 vaccine formulations each comprised of three peptides derived from either the extracellular portion of the molecule (n=28), the intracellular portion of the molecule (n=23), or, if they are HLA-A2, from three potential CTL epitopes (n=19). In an interim analysis of the first 22 patients to complete 6 vaccinations, 21 (95 %) developed Th immune responses to their immunizing peptide and 16 (73%) to HER2/neu protein as defined by a CD4 proliferation assay with a stimulation assay $\geq$ 2. For patients who responded, the stimulation indices to peptide and protein ranged from 2-35 and from 2-7, respectively. There have not been any predominant differences in the CD4 proliferative responses to protein among the different vaccine formulations. Time to development of immunity (SI$\geq$2) was a median of 3 vaccinations with a range of 1-6. Twenty of the 22 patients underwent DTH testing to their immunizing peptides. Thus, 60 DTH skin tests were placed with 28 positive responses (11, 5-9 mm and 17 $\geq$ 10mm). We have observed a significant relationship between the measured size of a DTH response and the peripheral Th stimulation index (SI). Moreover, biopsies at DTH sites have shown infiltration with CD3+ cells, HLA DR, a predominant CD4+ population and up-regulation of presumptive Langerhans cells. Toxicity was minimal with only three patients reporting measurable toxicity (2, grade 2 urticaria and 1, grade 1 myalgia). Specifically, no evidence of severe autoimmune phenomenon has been observed or reported in any enrolled patient.

The generation of HER2 protein specific responses was associated with epitope or determinant spreading in 18 (82%) of patients. The occurrence of epitope spread was significantly correlated to responses to protein (p-value<.0001). This phenomenon suggests that the immune repertoire to HER2 evolves during the course of vaccination. The development of a T cell response with a variety of specificities during immunization indicates that naturally expressed HER2 protein is being processed and presented in the MHC (see section E).

E.Flt3 Ligand (*FL*)

FL has been shown to be a potent stimulator of early hematopoietic cells, and in particular, of progenitor dendritic cells (DC)[10]. DC are the most potent APC in humans and comprise less than 10% of circulating WBC. Previous studies have demonstrated that FL administered systemically to mice can markedly increase circulating progenitor dendritic cells that retain antigen-presenting function and the capacity to stimulate the proliferation of antigen-specific T cells [11]. Administration of rhGM-CSF did not increase numbers of splenic DC suggesting a distinct effect of FL. Administration of FL alone, in fact, could confer resistance to a murine C3L5 breast cancer challenge [12]. Similarly, systemic administration to mice after challenge with B16 or EL-4 tumors resulted in significant decreases in tumor growth rates, with infiltration of dendritic cells noted within tumor tissue sections [13]. Importantly, adoptive transfer of spleen cells from Fl treated mice that rejected tumor challenge, mediated tumor rejection in recipient mice. CD8 depletion of the transferred cells did not confer protection while CD4 depletion of the transferred cells resulted in complete rejection of tumor growth in 3 of 4 mice and delayed growth of tumor in the remaining recipient [14]. Recombinant human FL has been safely given to normal human volunteers and has been used to expand human dendritic cells *ex vivo* from peripheral blood stem cells [15].

The ability of FL to stimulate the production of dendritic cells suggests it may be useful as a vaccine "adjuvant" by increasing the number of circulating and tissue-resident antigen presenting cells. The increased number of dendritic cells may thereby increase the presentation of a peptide vaccine antigen

and may promote a more vigorous T cell response. Matzinger has theorized that tolerence or activation to a peripheral antigen may not be determined by whether the antigen derives from self or non-self but by the conditions under which it is introduced, i.e., concentration of antigen, cytokine environment or presentation [16]. Autoimmunity is, in this paradigm, the consequence of determinant or epitope spread in which cytokines drive a cycle of up-regulation of antigen presentation. Thus, a virgin Tc encounters APC displayng peptide that is not normally part of its MHC-antigen profile. Thus, by increasing APC we may be able to increase presentation of epitopes of the self-protein HER2. The current study will evaluate the use of FL in this novel role as a potential vaccine adjuvant which by promoting the growth and differentiation of APC may result in increased HER2 specific T cell immunity.

4. Objectives

A. Primary Objective:

1. To determine the safety of administering Fl (+/- rhGM-CSF) with a peptide based HER2 vaccine administered to patients with breast or ovarian cancer whose tumors over-express HER2.

B. Secondary Objective:

1. To determine if FL + HER2 ICD peptide based vaccine (with or without rhGM-CSF) stimulates a greater antigen specific Th and/or CTL precursor frequency than what has been seen with the ICD peptides + rhGM-CSF as adjuvant alone.
2. To determine if the addition of Flt-3 ligand results in earlier generation of HER2 specific immunity (e.g. after 1 or 2 vaccines).
3. To see if HER2 specific CTL is preferentially generated in the presence of Flt-3 ligand.

5. Vaccine Preparation

A. HER2 derived peptides:

HER2 ICD Based Vaccine

p776-790	GVGSPYVSRLLGICL
p927-941	PAREIPDLLEKGERL
p1166-1180	TLERPKTLSPGKNGV

B. Peptide synthesis and characterization:

Peptides are provided by Corixa Corporation and were manufactured by Multiple Peptide Systems, San Diego, CA. using standard solid phase synthesis techniques employing Boc chemistry. Amino acids and resins for synthesis were purchased from Bachem California and the amino acids were analyzed by TLC and polarimetry prior to use. The peptides were cleaved from the solid support using anhydrous hydrogen fluoride and conventional techniques. Each peptide was purified by HPLC using a Waters Delta-prep system with a gradient of acetonitrile (containing 0.1% trifluoroacetic acid) in water (also containing 0.1% trifluoroacetic acid). The peptides were lyophilized to dryness before being passed over an anion exchange column to exchange the trifluoroacetate for acetate. GLP procedures were followed for the manufacture of these sequences.

The peptides were characterized by mass spectrometry to confirm their identity, by HPLC for purity using two different elution buffers, and by amino acid analysis for identity and peptide content. The water content of each peptide was determined by the Karl Fischer procedure and the data obtained used to verify the mass balance of the peptides.

C. Peptide formulation and stability:

Prior to formulation, the solubility of each peptide in 10 mM sodium acetate buffer (pH 4.0) was determined. Three mixtures of 3 or 4 peptides each were made up in a 10 mM sodium acetate buffer (pH 4.0), with the concentration of each peptide being 833 mcg/ml, to give a total concentration of 2.5-3.3 mgs/ml of peptide. This will allow the total vaccine dose to be 1.5-2.0 mg in 0.6 ml using 2 injections of 0.3 ml. The stability of each individual peptide in the acetate buffer, as well as the peptide mixtures, was monitored by HPLC and mass spectrometry. The dissolved peptides are stored at -20°C prior to use. The peptides were vialed under sterile conditions. Microbial and sterility testing were conducted to ensure safety.

D. rhGM-CSF:

rhGM-CSF (Sargramostim), supplied by Immunex Corporation, Seattle, WA, will be used as a vaccine adjuvant. rhGM-CSF is a growth factor that supports the survival, clonal expansion and differentiation of hematopoietic progenitor cells including dendritic antigen presenting cells. In preliminary animal experiments rhGM-CSF served as an effective adjuvant for the induction of antibody and CD4+ T cell responses [27]. The use of rhGM-CSF is associated with little toxicity [25,26,28]. rhGM-CSF is a sterile, white, preservative-free, lyophilized powder supplied in 500 μg-dose vials. Vials will be reconstituted with the peptide solution (already in a 1 ml volume of 10 mM sodium acetate buffer). Reconstituted rhGM-CSF (0.2 ml) with peptide solution (0.6 ml) will be injected i.d. for a total dose of 0.8 ml for each immunization. The volume of vaccine will not exceed 0.4 ml per each of 2 injection sites. Thus, for the current study, rhGM-CSF will be used at a total injection dose of 150 μg mixed prior to injection with peptide and injected intradermally at the time of immunization.

Recombinant human rhGM-CSF (rhGM-CSF), when administered intravenously or subcutaneously is generally well tolerated at doses ranging from 50 to 500 $\mu g/m^2$/day. Severe toxic manifestations are extremely rare in patients treated with rhGM-CSF.

Diarrhea, asthenia, rash and malaise were the only events observed in more than 5% of the rhGM-CSF group compared with the placebo group in phase III controlled studies of patients undergoing autologous bone marrow transplantation. In uncontrolled phase I/II studies for various indications, the adverse effects reported most frequently were fever, chills, nausea, vomiting, asthenia, headache and pain in the bones, chest, abdomen, joints, or muscles. Most of these systemic events reported with rhGM-CSF administration were mild to moderate in severity and rapidly reversed by the administration of analgesics or antipyretics. Other events reported infrequently were dyspnea, edema, local injection site reactions, and rash. Thrombosis and cardiac arrhythmia have also been reported, and there have been infrequent reports of tissue sloughing, leukemic progression, congestive heart failure, hepatomegaly, and intercranial bleeding; and isolated reports of Guillain-Barré syndrome and increased histiocytes. Neutropenia, although usually a pre-existing condition in the patients receiving rhGM-CSF, has been reported in association with administration. There was an increased frequency of severe thrombocytopenia in patients receiving concurrent chemotherapy and radiotherapy with rhGM-CSF. Eosinophilia and other blood abnormalities may occur.

There have been rare reports of 1) sequestration of granulocytes in the lungs with respiratory symptoms; 2) a syndrome characterized by respiratory distress, hypoxia, flushing, orthostatic hypotension, and partial loss of consciousness; 3) peripheral edema, pericardial or pleural effusions, and capillary leak of fluid; and 4) serious allergic or anaphylactic reactions.

Administration of rhGM-CSF may aggravate fluid retention in patients with pre-existent edema, capillary leak syndrome, or pleural or pericardial effusions. In some patients with pre-existing renal or hepatic dysfunction, elevation of the serum creatinine or bilirubin and hepatic enzymes has occurred during the administration of rhGM-CSF. Dose reduction or interruption of rhGM-CSF administration has resulted in a decrease to pretreatment values. Occasional transient and reversible supraventricular arrhythmia has been reported in uncontrolled studies, particularly in patients with a previous history of cardiac arrhythmia.

Stimulation of marrow precursors with rhGM-CSF may result in a rapid rise in white blood cell count. Dosing should be stopped if the ANC exceeds 20,000/cm^3. rhGM-CSF may stimulate the growth of myeloid malignancies; therefore, caution must be exercised in its use in these malignancies or myelodysplastic syndromes.

Because rhGM-CSF is only administered monthly in one arm of patients on the current protocol, most of these side effects are not anticipated. In our previous vaccine study using rhGM-CSF as an adjuvant in an identical fashion, we did see local skin reactions consisting of erythema and induration in 17% of the patients. This resolved without need for treatment over a 2-3 week period. We have also observed transient leukopenia within ½ hour of administration of rhGM-CSF which is a side effect well described and not associated with clinical sequelae. We and others have also noted the generation of a transient immune response to rhGM-CSF following its use in similar fashion, however no clinical sequelae were noted, and the significance of this observation is not clear at present.

D. FL:

FL, supplied by Immunex Corporation, Seattle, WA, will be used as a vaccine adjuvant. Previous studies in normal human volunteers have shown that doses up 100 µg/kg/d s.c. for 14 days were well tolerated [36]. Previous studies looking at the generation of dendritic cells showed that increased dendritic cells could be detected in the peripheral blood beginning shortly after daily administration, with peak levels of circulating dendritic cells seen on about day 9 of daily administration. Levels of peripheral dendritic cells remained elevated until approximately day 21 after cessation of treatment on day 14. For the current study, patients receiving FL will receive a daily dose of 20 µg/kg (up to a maximum of 1500 µg/day) administered subcutaneously daily for 14 days, followed by a 14-day rest period. FL is supplied as a sterile solution in 1500 µg single-use vials. Patients receiving FL will receive their first daily dose under observation, for training and instruction in self-administration.

Commonly expected side effects associated with FL administration have included erythema and induration at the site of injection. Bruising, pain, blistering and pruritis at the injection sites have also been reported. Increases in peripheral white blood cell counts, especially monocytes, are expected, as are increases in serum enzymes (such as LDH) that are associated with leukocytosis. Increases in liver function tests (AST and ALT) are less frequently seen. Enlarged, painful lymph nodes have also been reported. All of these side effects are expected and were predicted in animal models. They were reversible with discontinuation of the drug.

In addition, with FL there have been infrequent reports of back and muscle pain, malaise, nausea, diarrhea, paresthesias, pharyngitis, headache, rhinitis, herpes simplex stomatitis, dyspepsia, and hematuria. These events may or may not have been related to the drug. Side effect reports following the administration of FL in combination with G-CSF or RHGM-CSF have included constipation, albuminuria, emesis, conjunctivitis, dizziness, sweats, and rare cases of hypercoagulability. These events may or may not have been related to FL. Although there is no evidence in animal models that healthy

animals develop autoimmune disease following FL administration, it is unknown whether FL may exacerbate autoimmune disorders. Therefore, subjects with a personal history of autoimmune disease will not be enrolled to receive FL.

Furthermore, some drugs used to treat cancer have an inherent risk of causing secondary cancers and/or leukemias. Although there is no evidence to date that FL has an inherent risk of causing such problems, because it is a growth factor for hematopoietic progenitor cells this remains a theoretical risk. Likewise, because of the investigational nature of this drug, there may be other unanticipated side effects that have not yet been reported.

6. Patient Selection

A. Inclusion Criteria

1. Patients must have:
   a. Stage III breast or ovarian adenocarcinoma that overexpresses HER-2 and has been treated by surgical resection, conventional chemotherapy or radiation therapy as appropriate.

or

   b. Stage IV breast or ovarian adenocarcinoma that overexpresses HER-2 and is stable or in complete remission such that the patient is expected to remain off chemotherapy for the entire trial (7 months).
2. Patients must have documented HER2 protein overexpression in their tumor (either primary tumor or metastasis).

3 Patients must be off cytotoxic chemotherapy and/or treatment dose corticosteroids for at least one month prior to enrollment. Patients cannot concurrently be receiving Herceptin or other immune modulators.

4. Patients should have an expected survival of at least 12 months.

5. Patients must have a Karnofsky score of 90% or greater (Appendix A).

6. Female patients should be done with child-bearing. Male patients must agree to use birth control methods to prevent pregnancy during the study period.

7. Patient's total WBC must be greater than 3500 mm^3, platelet count greater than 100,000 mm^3, serum creatinine less than 1.5 mg/dl or a creatinine clearance greater than 60 cc/min, serum bilirubin less than 1.5 mg/dl.

8. Age $\geq$ 18 years old.

Patients must meet all of the criteria listed to be eligible for enrollment into the study.

Exclusion Criteria

1. Patients with a previously diagnosed or known history of an auto-immune disease
2. Concurrent enrollment on other Phase I studies.

7. Experimental Design

There will be 2 study arms with 5 patients enrolled in each arm for a total of 10 patients.
Patients will be vaccinated monthly for six months. All patients will self administer FL @ 20 mcg/kg/day (maximum dose 1500 mcg/day) s.c. for the 1st 14 days of a month followed by 14 days of rest. Vaccinations will be administered on day 7 of FL. This cycle will define a vaccine cycle and will be repeated evry 28 days (monthly) for 6 consecutive months.

ARM 1: ICD peptides + rhGM-CSF (150 mcg) mixed together and given as a single i.d. injection day 7 of FL administration.

ARM 2: ICD peptides given as a single i.d. injection on day 7 of FL administration.

Patients will be assigned to arms 1 or 2 in alternating sequence. In other words, the first patient enrolled will be assigned to arm 1, the second to arm 2, the third to arm 1, etc..

All patients will undergo a 200 cc blood draw at beginning, after the 3rd and after the 6th vaccinations to insure adequate PBMC for the laboratory component of the study. Chemical and clinical parameters will be evaluated monthly to determine systemic toxicity.

8. Criteria for Premature Study Termination

Patients will be enrolled on the study as outlined in section 7. If at any time during the study, there is suffficient evidence suggesting that the Grade 3 or Grade 4 toxicity rate is excessive, then the study will be terminated. ARM 1 and 2 will be evaluated as separate populations. Toxicity will be measured by the modified NCI Common Toxicity Criteria set in Appendix B

An excessive Grade 3 toxicity rate will be taken to be 20% and an excessive grade 4 toxicity rate will be taken to be 10%. An exception will be made for grade 3 skin toxicity, as generalized rash with some mild symptoms are expected in patients treated with FL and local DTH rsponses are expected from vaccination. Therefore, grade 3 skin toxicity will not used as stopping criteria. Evidence that the toxicity rate is excessive will be considered sufficient if the lower limit of the 90% one-sided confidence interval for the estimate of the true toxicity rate exceeds the appropriate limit (20% for Grade 3, 10% for Grade 4). Operationally, this will occur if any of the following occur:

Grade 3 toxicity: Present in 2 of 2 patients, 3 of 5 patients.
Grade 4 toxicity: Present in 1 of 1 patient, 2 of 5 patients.

9. Management of Toxicities and Complications

If a patient develops grade 3 toxicity or greater related to the immunizations, no further vaccinations will be given to that patient. If the toxicity observed is felt to be related to the immune response generated, a regimen of corticosteroids will be administered. The following dose schedule will be used:

Day 1: Intravenous Solu-Medrol at 1 mg/kg IV q12 hr
Day 2: Intravenous Solu-Medrol at 1 mg/kg IV x 1
Day 3-4: Prednisone at 30 mg p.o. bid
Day 5-6: Prednisone at 15 mg p.o. bid

Day 7-8: Prednisone at 10 mg p.o. bid
Day 9-10: Prednisone at 10 mg p.o. qd
Day 11-12: Prednisone at 5 mg p.o. qd

Although unexpected, any patient requiring IV corticosteroids will be monitored on the CRC unit of the University of Washington Medical Center until resolution of symptoms.

10. Plan of Treatment

The following section describes the schedule for initial H&P, vaccine inoculations, clinical and laboratory evaluations. When a chemistry panel is indicated the following tests are performed: sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, SGOT, SGPT, alkaline phosphatase, and LDH.
This plan is summarized in Appendix C.

All eligible patients will undergo:

A. Initial Evaluation:

1. Sign consent form.
2. Medical history and complete physical examination. Clinical evaluation, including vital signs symptoms assessment, and Karnofsky score (Appendix A).
3. 200 cc blood draw procedure (heparanized blood) to obtain PBMC for the evaluation of preexisting cellular immunity to the HER2 protein, peptide and adjuvant by T cell proliferative assays and by CTL CRA. PBMC will also be used to assess precursor frequencies of HER2-specific CTL by ELISPOT. (Appendix D)
4. Obtain 10 ml. of peripheral blood for the evaluation of a preexisting antibody response to HER2 protein, peptide and the adjuvant.
5. Evaluation of blood cell counts, chemistry panel, and urinalysis.
6. DTH skin testing using sterile water, adjuvant, individual ICD peptides, and a panel of standard DTH antigens as baseline assessment. Skin biopsy of baseline positive responses.
7. Patients will be assigned in alternating fashion to one of the two treatment arms, as documented above.

B. Monthly immunizations:

1. Monthly brief physical examination, with symptom assessment, toxicity assessment and Karnofsky score.
2. Patients will receive monthly immunizations as described in section 7 above. Specifically, if on arm 1 patients will receive the three ICD peptides mixed and administered i.d.. on day 7 of FL administration. If on arm II, patients will receive the three ICD peptides mixed with 0.2 ml of reconstitued rhRHGM-CSF (150 mcg) administered i.d.. The volume of vaccine administered will not exceed 0.4 ml. and where the mixture is greater than 0.4 ml it will be divided into two equal doses which will be administered within 4 cm. Of each other. Patients will be seen on the first day of the monthly FL administration and on the day of peptide vaccine administration.
3. Following vaccinations, the subjects will be observed for 60 minutes. Temperatures and reactions will be assessed by the staff at the end of the post immunization period. Subjects will take and record their temperatures the same evening (and at additional times if they have symptoms of fever) and record any other symptoms they experience. DTH responses will be assessed at 24-48 hours. Skin changes will be reviewed and documented at each visit.

4. Monthly CBC, chemistry panel (to be obtained on the first day of each 28-day cycle) and urinanalysis.
5. On the first day of the third immunization cycle, 210 cc of blood will be drawn to prepare PBMC and sera for the evaluation of augmentation or generation of an immune response to HER2. Specifically, T cell proliferative assays will be used to evaluate a T cell response to HER2 protein or peptides, and ELISA will be used to detect an antibody response to HER2 protein and peptides. (see section Initial Evaluation)

D. Final Evaluation:

1. 48-hour DTH skin testing using sterile water, adjuvant, and peptides, with skin biopsy of DTH responses.
2. 200 cc blood draw procedure to obtain adequate numbers of PBMC for the evaluation of elicitation or augmentation of cellular immunity to the HER2 protein, peptide and adjuvant by T cell proliferative assays and standard CTL CRA, and to assess precursor frequencies of HER2-specific CTL by ELISPOT.
3. Obtain 10 ml of peripheral blood for evaluation of antibody responses to HER2 or adjuvant.
4. Evaluation of blood cell counts, Chem 12 and urinalysis.
5. Where feasible, obtain 200-ml blood samples for the preparation of PBMC and sera every 2-3 months for up to one year following the last vaccination to evaluate retention or augmentation of cellular and humoral immunity.

11. Immunologic Monitoring

See Appendix D

12. Reporting Adverse Events

A. Definition:

A life-threatening event is defined as the patient, in the view of the investigator, having been at immediate risk of death from the reaction as it occurred. It does not include a reaction that, had it occurred in a more serious form, might have caused death. All adverse events that do not meet at least one of the criteria listed below are defined as non-serious.

1. Patient death
2. Life-threatening event
3. Persistent or significant disability/incapacity
4. Prolonged hospitalization or requirement for hospitalization during the treatment and monitoring period related to the vaccinations given.
5. An important medical event that, based on appropriate medical judgment, may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

B. Procedure for Reporting Adverse Events: